DETECTION OF AIRBORNE TRICHOTHECENE MYCOTOXINS FROM *Stachybotrys chartarum* AND THEIR RELATIONSHIP TO SICK BUILDING SYNDROME

by

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A DISSERTATION

IN

MEDICAL MICROBIOLOGY

Submitted to the Graduate Faculty of
Texas Tech University Health Sciences Center
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

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December, 2004

ACKNOWLEDGEMENTS

I would like to express my gratitude to my mentor and inspiration Dr. David Straus who taught me the intricacies and discipline of being a scientist. From my first days in the lab which were full of follies ("learning experiences") to the major accomplishments I present here, Dr. Straus has always been by my side. Dr. Straus, I hope you enjoy ***these*** data.

I would also like to individually recognize my laboratory coworkers without whom this would have been an impossible task. I thank Dr. Stephen Wilson for his continual scientific devotion and enthusiasm for the field. He was always available to answer questions and offer support. I would like to show my appreciation to Dr. David Douglas for teaching me the intricacies of chromatography, a delicate and enjoyable field of study. Dr. Douglas also taught me not to immediately take data at face value, a quality which will no doubt be invaluable for my future career. I also thank Ms. Enusha Karunasena whom I have known since my first day in the laboratory. She and I have been on this adventure together from day one. To Mr. Jared Martin, you and I have known each other for over 15 years and having you in the laboratory has been essential for completion of this project. You have always been there for my personal and professional lives and for that I cannot thank you enough. I would like to express my thanks to Mr. Curtis Carriker, Mr. Matt Fogle and First Lieutenant Colby Layton for their scientific eagerness and contributions to my project. Finally, I would like to recognize Dr. Danny Cooley (whom we all miss), Dr. Chris Schwab, Dr. Chunfa Wu, Dr. Larysa Andriychuk, Mr. Gary Fortenberry, and Mr. Cameron Gulley who all offered their unique skills to this project.

I would like to express my gratitude to my committee members, Drs. Joe Fralick, Abdul Hamood, Jim Hutson, and Howard Strahlendorf for their guidance and distinct perspectives concerning this project.

Special thanks are due to the faculty and staff of the Department of Microbiology and Immunology at Texas Tech University Health Sciences Center. I would like to specifically recognize our chairman Dr. Ronald Kennedy for welcoming me into his personal and professional life and for devoting his precious time to ensure my future success. Additional thanks are owed to all of the graduate students whom have become more my friends than classmates over these last five years. Particular thanks are due to Mr. Landon Westfall and Mr. Andy Schaber who could always find the time to help me out with those "tricky" molecular problems.

I am grateful to Bruce Ferguson and EnviroLogix and to Dick Jarman and Sceptor Industries for their valued support and friendship over the years. Additional thanks are due to Drs. Andrew Campbell, Jordan Fink, and Aristo Vojdani who played a key role in our human serum studies. I would also like to recognize all of the home and building owners who kindly allowed us into their dwellings to complete this research.

I wish to recognize my family whose love and encouragement allowed me to reach the end of this journey. To my parents, I cannot thank you enough for your support over the years. You have been the perfect role models and, through your own experiences, taught me the importance of a higher education. Seeing the proud look on your faces is a sign that I have truly accomplished my goals. To my sisters Kara and Heather and their families, you were always there to help out your little brother no matter what the issue and for that I am ever grateful. To Jimmy and Debra Kilgore, who welcomed me into their family during the course of this project, your kind and generous nature have helped me reach the finish line, a gift that will forever be remembered.

Finally, to my loving wife Kristi, I owe a debt that can never be repaid. Her strength and motivation transformed this seemingly impossible task into a challenge that I overcame and, am proud to say, could not have been completed without her. I devote all of my hard work to her and our future together. Hey Kristi, you know what? Yeah...me too.

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ABSTRACT

The growth and propagation of fungi in water-damaged buildings has long been recognized as a potential health risk to occupants of such environments. Reported symptoms from inhabitants of these "sick" buildings range from allergic rhinitis, headaches and watering of the eyes to more severe symptoms that warrant considerable concern such as hemorrhaging, nausea/vomiting/diarrhea, dizziness, and loss of mental capacity. Of the fungi capable of contaminating indoor environments, *Stachybotrys chartarum* is thought to pose the most significant human health risk. *S. chartarum* is a known producer of many compounds that have the potential to adversely effect occupant health, the most noteworthy being the highly toxic macrocyclic trichothecene mycotoxins. Currently, the relationship between the presence of trichothecene-producing strains of *S. chartarum* in water-damaged buildings and adverse human health effects is unclear. This is primarily due to the lack of data showing the co-presence of this organism and its mycotoxins (in an airborne state) in such environments.

In this study, we present evidence that *S. chartarum* trichothecene mycotoxins become airborne and exist in buildings contaminated with this organism. Under controlled situations, we were repeatedly able to separate and collect airborne particulates originating from *Stachybotrys* growth. Our results demonstrated that airborne trichothecene mycotoxins were present on large, poorly respirable particles such as intact conidia, as well as highly respirable particulate matter such as fungal fragments. Furthermore, we were able to collect airborne trichothecene mycotoxins in natural indoor environments contaminated with *S. chartarum*, one in which separation and collection of

particles was done as in our controlled setups. Concentrations ranged from less than 10 to greater than 1000 pg/m^3 of sampled air.

In addition, we present data demonstrating that these compounds can be detected in sera from individuals with known mold (specifically *S. chartarum*) exposure. Overall concentrations were low with the exception of two samples that demonstrated uniquely high values (43 and 84 ng/ml) indicating a definitive exposure. When looked at in total, our data show that airborne trichothecene mycotoxins can be isolated in *Stachybotrys chartarum*-contaminated buildings and, based on their detection in human serum samples, may represent a significant occupant health risk.

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CHAPTER I

INTRODUCTION

For centuries mankind has postulated that exposure to poor quality indoor air (associated with damp and moldy environments) may lead to detrimental health effects. One of the earliest known documentations concerning the negative effects of fungi in the indoor environment is from the Book of Leviticus:

> On the seventh day the priest shall return to inspect the house. If the mildew has spread on the walls, he is to order that the contaminated stones be torn out and thrown into an unclean place outside the town. If the mildew reappears in the house after the stones have been torn out and the house is scraped and plastered, it is a destructive mildew and the house is unclean. It must be torn down – its stones, timbers, and all the plaster – and taken out of town (Leviticus 14: 39-47).

Since this biblical depiction, the scientific research attempting to relate indoor mold growth and adverse human health effects has been extensive and is ever-growing. The awareness of fungal-contaminated buildings has existed for a long time, but only recently has the problem escalated beyond control. There are several hypothesized reasons for this, each of which likely contributes to the overall dilemma. First, modern communication technology has allowed information concerning indoor fungal growth to spread in a rapid manner. It is thought that the problem has existed for longer than once believed, but it has just recently been recognized as a major concern. Second, modern living habits have changed dramatically whereby the majority of one's time (upwards of

90%) is spent indoors (102). Combined with poor indoor conditions (such as mold contamination), an individual is more prone to experience adverse health effects. Perhaps the most important reason for the problem of indoor fungal growth is the manner in which modern buildings are constructed. In 1973, during the midst of extreme increases in inflation rates in the United States and Western world, the Organization of Petroleum Exporting Countries imposed an oil embargo shortly following a U.S.-aided Israeli counterattack on the Egyptian army. This embargo was meant as an attack on the West and resulted in a 70% increase in the price of oil. This was extremely detrimental because, at the time, the U.S. was importing some 35% of its energy needs with petroleum reserves nearly depleted. Economically, the U.S. suffered greatly. As a result, in order to preserve energy (and money), building construction became increasingly conservative. Cheaper building materials were implemented and such structures were no longer constructed to allow for fresh (outdoor) air circulation. These "tight" buildings had systems setup whereby the same air was constantly re-circulated throughout the day. When a problem such as a water leak arose and went unchecked, there was a high probability for microbial (fungal) growth to arise. Combined with cheap building materials, poor air ventilation and circulation, etc., adverse human health effects began to appear with increased frequency. These same types of issues exist today and have resulted in billions of dollars in insurance and health claims (7). Unfortunately, significant efforts into researching preventative measures have not been conducted until recently (21, 46, 63, 99, 109).

To date, the establishment of a definitive causal relationship between fungal growth in buildings and occupant illness has been difficult (66). One of the main reasons

for this is that indoor air is dynamic in nature and is composed of a variety of pollutants, all of which may negatively affect human health. Some of these pollutants include volatile organic compounds (VOCs) such as toluene, benzene, alkenes, aromatic hydrocarbons, alcohols, combustion gases, carbon dioxide, ozone, formaldehyde, and even radon (64, 73, 83, 94). Problems associated with poor indoor air quality (IAQ) have also been linked to activities such as photocopying, video terminal exposure (54) and smoking (75). Additionally, numerous studies have suggested that organic and inorganic debris (such as allergens and dusts, respectively), ventilation rates, temperature, noise levels, and the overall quality of the work environment are important factors (5, 13, 21, 48, 91). Psychosocial issues, particularly with the current widespread media exposure of indoor molds, may also play a key role (8, 42, 69, 93). Each of the above-mentioned factors has the potential to result in symptoms associated with the phenomenon referred to as "sick building syndrome" (SBS). These commonly consist of complaints associated with upper respiratory distress such as headaches, eye irritation, nasal and sinus congestion, cough, and overall "cold and flu-like" symptoms. The most accepted and researched reasoning behind SBS has been microbial in nature. Fungi and associated mycotoxins have been the specific focus because of the numerous serious symptoms (such as pulmonary hemorrhage and neurological disorders) that have been reported in association with such exposures (see below).

Microbes are ubiquitous in indoor environments. Perhaps one of the most recognized and important cases entailing indoor microbes and human health occurred during a celebration of the United States' bicentennial in July of 1976. Two days after the start of the celebration, a mysterious pneumonia illness began to overcome a large

number of individuals. The majority of the cases were isolated to members of the American Legion whom had been celebrating the event in the Bellvue-Stratford Hotel in Philadelphia. The illness had eventually affected 221 patients of whom 34 died. The Centers for Disease Control and Prevention was able to pinpoint the cause as a bacterium which thrived in the cooling tower of the hotel and had been dispersed via the building's air conditioning system. The responsible bacteria were eventually named *Legionella* after those who contracted the disease. Bacteria have long been known to be problematic in indoor environments as agents of infection and endotoxin-related illness (4, 44, 52). However, it is dampness and accompanying fungal growth that have been consistently associated with SBS and thus a major focus of attention (19, 23, 44, 67, 72, 109).

Fungi are eukaryotic, unicellular, dimorphic, or filamentous and are usually spore-bearing organisms. There are more than 100,000 recognized species of fungi, comprising 25% of the biomass of the earth (82). They are ubiquitous in nature, being present outdoors and indoors at all times. Many studies have been conducted to ascertain the mycological composition of indoor and outdoor environments. Outdoor air varies in its fungal makeup depending on the geographic region. In one study performed in the United States, five fungal genera were consistently found in the outdoor air and comprised 95% of the identified fungi. These genera were (in order of prevalence) *Cladosporium*, *Penicillium*, *Chrysosporium*, *Alternaria*, and *Aspergillus* (19). These results are consistent with those obtained by others (98). The fungal composition of indoor air is usually similar in non-problematic buildings. The same genera can also be isolated in problematic (water-damaged) buildings, though these "sick" buildings will often contain higher counts of these organisms and additional isolates such as the genera

Chaetomium and *Stachybotrys* that are rarely isolated from the outdoor air. Perhaps the most important difference between indoor and outdoor air is that the composition of outdoor air constantly changes over a given time period. Conversely, the makeup of indoor air tends to remain unchanged (72). This can be detrimental to occupants, particularly if the environment is contaminated with a mycotoxin producing organism like *Stachybotrys.*

Adverse human health effects resulting from fungal exposure can fall into three major categories: allergy, infection, and toxicity. Allergic manifestations of airborne fungi have been well-studied in animal models and humans and include asthma and its exacerbation, rhinitis, allergic sinusitis, hypersensitivity pneumonitis, and various other inflammatory responses (18, 25, 39, 52, 85, 96). These types of responses have often been reported in occupants of mold-contaminated buildings (32, 40, 55, 67). Regarding infection, most fungi are not pathogenic to humans. Immunocompromised individuals are at the greatest risk for fungal infections (1, 6, 26, 51, 70). There are, however, fungi capable of infecting immunocompetent individuals. These include dermatophytes, *Blastomyces*, *Cryptococcus*, *Histoplasma*, *Coccidiodes*, and *Candida.* Although fungal infections have been attributed to mold-contaminated indoor environments (111), they are generally not considered to be a part of the symptoms associated with SBS. Of the above-mentioned ways in which fungal exposure can affect human health, toxicity is the most poorly understood as well as the most difficult to establish, specifically in context of mold-contaminated indoor environments.

Most fungi produce some type of toxic secondary metabolite, or mycotoxin. Mycotoxins are defined as natural products produced by fungi that evoke a toxic response

when introduced in low concentration to higher vertebrates and other animals by a natural route (11). The earliest recorded cases of human illness resulting from mycotoxins date to the Middle Ages with an ailment known as "St. Anthony's Fire." This was primarily a disease of the poor and, in the 18th century, was discovered to be due to ingestion of *Claviceps purpurea*-contaminated rye. Commonly known as ergotism, ingestion of *Claviceps* and its associated mycotoxins (also known to be produced by some species of *Penicillium*, *Aspergillus*, and *Rhizopus* (86)) can cause vasoconstriction, gangrene, and convulsions. Since the discovery of ergot poisoning, knowledge concerning mycotoxins in agriculture (animal and human foodstuffs) and their effect on health has been extensive (10, 14, 45, 53, 74). However, it is inhalation exposure rather than ingestion of mycotoxins in buildings that is of concern. Inhalation, as a route of exposure, has been shown to be significantly more toxic than oral dosing (108). To date, the relationship between mycotoxins in mold-contaminated indoor environments and occupant health has not been clear. It is well accepted, however, that a means to detect indoor mycotoxins and evaluate human exposure is much needed (56, 66, 84, 100).

Buildings have evolved into man-made ecosystems whereby a limited number of fungal species will dominate, depending on water activity (a_w) and nutrient availability (34, 79). Water activity is the ratio of water vapor pressure of a material or food to that of pure water at the same temperature. This is not to be confused with relative humidity, which is the ratio of vapor pressures of solution and pure water that is expressed as a percentage (16). Many instances of mold growth in buildings follow a general sequence of events: building failure, moisture penetration, moisture damage, mold growth, and occupant illness (99). Grant et al. divided indoor molds into three groups primarily based

on a_w (41). Primary colonizers grow best under a water activity of less than 0.8 and include various species of *Penicillium* and *Aspergillus*, *Eurotium*, *Paecilomyces*, and *Wallemia*. Among these, *Penicillium* and *Aspergillus* are the predominant genera (79). Species of *Penicillium* are known to produce such toxic compounds as mycophenolic acid, patulin, citrinin, and chaetoglobosins (12, 29, 38). Among these, patulin is thought to be the most toxic (in relevance to indoor environments) as it has demonstrated inhibitory effects upon the activity of mouse peritoneal macrophages (15, 79). Additionally, *Pencillium expansum* isolates have been shown to produce patulin on wood, one of the most extensively used building materials. The chaetoglobosins (also produced by *Chaetomium globosum*; see below) are hypothesized to be important contributors to IAQ problems, but current research concerning their significance to occupant health is lacking. Species of *Aspergillus* are known to produce a variety of mycotoxins, the most recognized being aflatoxin B_1, gliotoxins, ochratoxin A, and sterigmatocystin (38, 106). Though frequently isolated from mold buildings, many species of *Aspergillus* produce limited amounts of (or do not produce) toxic metabolites on building materials (78, 89). Nonetheless, toxins produced by these organisms have been isolated from indoor environments, thus demonstrating their potential as human health risks (31, 92). Mycotoxins produced by the remaining mentioned primary colonizers have been poorly researched, but based on existing literature, are not thought to be associated with SBS.

Secondary colonizers require a minimal a_w between 0.8 and 0.9 and include species of *Alternaria*, *Cladosporium*, *Phoma*, and *Ulocladium*. As a whole, this group of organisms does not produce the potent mycotoxins that are hypothesized to be associated

with adverse human health effects in buildings. Some species of *Alternaria* produce alternariol and/or altenuene (2, 22), but these are considered to have a low toxicity value (79). *Cladosporium* has been shown to produce compounds of low *in vitro* toxicity. However, this organism is extremely common and existing literature suggests that it does not induce toxigenic effects on humans.

Tertiary colonizers are known as water-damage molds (requiring $a_w > 0.9$) and include the following organisms that are highly associated with toxicity and human health in mold-contaminated indoor environments: *Chaetomium globosum*, *Memnoniella echinata*, *Stachybotrys chartarum*, and various species of *Trichoderma*. *C. globosum* is one of the most common fungal isolates from problem buildings (87). It is known to produce the highly cytotoxic chaetomins and chaetoglobosins that have been shown to inhibit cell division and glucose transport (87, 97). Other species produce sterigmatocystin (97), but they have not been found in buildings (79). Nielsen et al., employing high performance liquid chromatography, demonstrated that *C. globosum* produces large quantities of chaetoglobosins A and C on wallpapered plasterboard and other common building materials (80). The direct contributions of this fungus and its associated mycotoxins to SBS have yet to be examined. *M. echinata* produces a variety of mycotoxins including griseofulvin, spirocyclic drimanes, and two trichothecene mycotoxins, trichodermin and trichodermal (59, 60). Trichothecenes are perhaps the most important class of mycotoxins in context of poor IAQ and SBS and are discussed in further detail below. Reportedly six species of *Trichoderma* have been isolated from indoor environments (68). This genus is known to produce an assortment of mycotoxins including trichodermol and trichodermin (trichothecenes), gliotoxin, and others.

However, existing data suggest that toxin (particularly trichothecene) production by *Trichoderma* is limited on building materials and, consequently, in indoor environments (68, 81). Of all the fungi isolated from water-damaged indoor environments, *S. chartarum* is hypothesized to pose the greatest occupant health risk. It is known to produce some of the most toxic compounds ever isolated from a fungus. Because of its recognized immunosuppressive nature and toxicity, *S. chartarum* has been the key focus of IAQ investigations and scientific research.

Stachybotrys chartarum (formally *S. atra* and *S. alternans*) is a filamentous fungus belonging to the family *Dermatiaceae* (Figure 1.1). It produces simple or branched conidiophores approximately 100 μm tall (up to 1000 μm) and 3-6 μm wide. Conidiophores are hyaline or grayish early in development and become olivaceous brown to black at later stages. These organisms bear clusters of 4-10 ellipsoidal phialides from which conidia bud. Conidia are produced in slimy heads, 7-12 x 4-6 μm, and are initially hyaline, becoming dark olivaceous-gray over time. They vary in texture from smooth-walled in early development to coarsely roughened and warted later in maturity. Organisms belonging to this genus are cellulolytic saprophytic organisms with a worldwide distribution (9). In 1837, Corda first defined the genus *Stachybotrys* from a strain growing on domestic wallpaper in Prague (20). One of the earliest described pathogenic roles for *Stachybotrys* originated in the Ukraine in the early 1930s. During that time, a unique disease of horses was recognized that was characterized by lip edema, stomatitis, oral necrosis, rhinitis, and conjunctivitis. These symptoms often progressed to the eventually death of the animal (36). A rare "atypical" form of the disease also existed in which severe neurological symptoms such as loss of motor reflexes, hyperirritability,

blindness, and stupor were recorded. This form was highly fatal and pathologic examination revealed massive hemorrhage and necrosis throughout the alimentary tract. Severe lung congestion and edema were also noted (107). In 1938, this disease was associated with *Stachybotrys*, but it was not until ten years later that the etiologic organism was identified in contaminated grain (to which the animals were exposed) as *S. alternans* (107). In the 1940s, Drobotko et al. carried out a study in which *Stachybotrys*-contaminated straw was fed to horses. The animals subsequently developed an illness similar what was described in the Ukraine ten years earlier. At this same time, it was discovered that this disease was caused by toxins from the fungus and that isolated toxins could produce the same symptoms (36). Drobotko et al. defined this disease as "stachybotryotoxicosis" which was also being reported in other parts of the USSR and Eastern Europe (37, 79). Similar cases have been reported in animals in recent years as well (43, 95, 101). These toxigenic effects seen in animals gave rise to the idea that *Stachybotrys* could elicit similar effects in humans.

In those areas where stachybotryotoxicosis was reported in horses and other animals, some humans, especially those who handled the contaminated fodder and had close contact with moldy straw, developed significant dermatologic and respiratory symptoms (28, 36). Respiratory symptoms included angina, bloody rhinitis, cough, throat pain, chest tightness, fever, and occasional leukocytopenia. Regarding dermatologic symptoms, Dill et al. recently described the development of painful inflamed efflorescences on the finger-tips of three women who were handling *S. chartarum*-contaminated decomposable plant-pots (27). To support the hypothesis that *S. chartarum* may be responsible for symptoms associated with SBS, numerous controlled

experiments using animal (mainly murine) models have since been performed (35, 71, 88, 110, 113). Data from these studies showed the predominant effects from exposure to be brochoconstriction, cytotoxicity, hemorrhage, inflammation, lung convertase alteration, and pulmonary irritation. These responses are not unlike what has been reported in humans exposed to *S. chartarum* in indoor environments. In 1986, Croft et al. published one of the first case reports suggesting airborne toxicosis due to *S. atra* in a residential building. Although no clinical abnormalities were noted by physicians and no air samples were taken, the "recurring cold and flu symptoms, sore throats, diarrhea, headache, fatigue, dermatitis, intermittent focal alopecia, and general malaise" reported by the inhabitants suggested a *Stachybotrys* exposure. Since this report, numerous other investigations have been conducted that relate the presence of *Stachybotrys* to such occupant health effects as respiratory and central nervous system disorders (62), acute pulmonary hemorrhage (17, 24, 30, 76), neurological impairment (90), and general pulmonary disease (49, 50). Although never directly demonstrated, the authors of these studies concluded that airborne *S. chartarum* mycotoxins were the causative agents of the observed symptoms.

As already mentioned, toxin production by *S. chartarum* was recognized as early as the 1940s. Because these mycotoxins are thought to play a key role in SBS, they have been the focus of numerous rigorous chemical and biological analyses. Some of the most cytotoxic fungal metabolites discovered have been isolated from *S. chartarum* (57). The products of most interest are the macrocyclic trichothecene mycotoxins, although *S. chartarum* can produce other compounds of clinical importance including the immunosuppresent phenylspirodrimanes and dialdehydes (58). Recently, detailed

analyses of several cultures of *S. chartarum* showed that some isolates do not produce macrocyclic trichothecenes, but rather a group of compounds named atranones (47). Toxicologic examination of the atranones has yet to be performed. These "non-toxic" strains of *Stachybotrys* (now classified as a different species (3)), do not induce the same toxin-associated responses observed with the macrocyclic trichothecene-producing *S. chartarum* (65). Because of their highly toxicologic nature, the trichothecenes have received the most attention and thus are the primary concern for potential exposure-related problems in mold-contaminated buildings.

The trichothecenes are a group of related mycotoxins with varying degrees of toxic potential that is dependant upon their structural composition (103). All trichothecenes possess a sesquiterpenoid ring system and contain an epoxide at the $C_{12,13}$ position that is responsible for the majority of their toxicity (Figure 1.2). These toxins can be classified according to the presence or absence of specific functional groups (100). Types A and B trichothecenes are distinguished by the absence or presence of a carbonyl group at the C_8 position, respectively. Type C trichothecenes possess an additional epoxide group at the $C_{7,8}$ or $C_{9,10}$ position. Type D, or macrocyclic, trichothecenes contain an additional cyclic ring between the $C_{4,15}$ positions. Examples of types A-C are shown in Figure 1.3. Selected macrocyclic trichothecene mycotoxins (produced by *S. chartarum*) are shown in Figure 1.4. Biochemical approaches to the mode of action revealed that these toxins are potent inhibitors of protein synthesis in eukaryotic cells. Specifically, trichothecenes interfere with the active center of peptidyl transferase of the eukaryotic 60S ribosomal subunit thereby inhibiting peptide initiation, elongation, and/or termination reactions (33, 105). Described secondary effects include inhibition of DNA

synthesis and disruption of cellular membrane integrity (33). Trichothecenes have been implicated in human illness dating back to the 1930s when an epidemic of alimentary toxic aleukia (ATA) in humans was described in the former Soviet Union (61). ATA occurred under conditions of near famine where individuals were forced to consume over-wintered grain that was contaminated with species of *Fusarium*. One of the isolated species, *F. sporotrichioides*, was found to produce high amounts of the Type A trichothecene T-2 toxin. Symptoms of ATA ranged from the early stages of severe gastrointestinal illness to the late stages of necrotic lesions and hemorrhagic complications. The highly cytotoxic effects of trichothecenes that were observed during the ATA epidemic led researchers to evaluate their potential as a cancer treatment. Diacetoxyscirpenol, a Type A trichothecene also known as anguidine, was investigated as a chemotherapy drug in patients with advanced forms of cancer (77, 112). Unfortunately, adverse health effects were reported in the individuals including nausea and vomiting, fever and chills, hypotension, skin erythema, confusion, and lethargy. Overall, myelosuppression was minimal. Trichothecenes have also been investigated in a controversial issue regarding their misuse as agents of biological/chemical warfare. Allegedly, in the late 1970s and early 1980s, trichothecenes were aerosolized over civilian and refugee populations in Laos, Kampuchea, and Afghanistan. Symptoms associated with exposure to this "yellow rain" included bleeding, nausea, fever, dyspnea, dizziness, and vertigo (104, 108). The most recent implications concerning trichothecene mycotoxins and human illness have been in *S. chartarum*-contaminated buildings. The toxic nature of *S. chartarum* and its associated macrocyclic trichothecene mycotoxins strongly supports the hypothesis that exposure in a closed building environment

contaminated with this organism is a health risk to occupants. Current data to substantiate this claim are lacking. To our knowledge, the existence of airborne *S. chartarum* trichothecene mycotoxins, particularly conidia-free, has never been demonstrated in natural mold-contaminated indoor environments. Furthermore, methods to evaluate human exposure to these mycotoxins are extremely limited. To address this research gap, my dissertation work was divided into three phases. Because of their physical properties, low quantities of *S. chartarum* conidia are found in indoor environments. Furthermore, due to their shape, size, and conglomerative nature, the conidia are not apt to reach the lower extremities of the human respiratory tract (i.e., they are cleared rapidly) and cause the toxic ailments commonly associated with SBS. These facts led into the first phase of my research which was to determine if *S. chartarum* trichothecene mycotoxins could become airborne on particulates smaller than conidia (i.e., highly respirable particles). Secondly, most of the research claiming to find mycotoxins in indoor environments has focused on fungal-contaminated bulk materials and not airborne concentrations. Assessing the presence and concentration of mycotoxins on any given piece of material is not a reliable means of human exposure. Therefore, the second phase of my research was to develop new sampling methods and determine the presence of *S. chartarum* trichothecene mycotoxins in native mold-contaminated buildings. As mentioned, virtually no studies have been conducted that evaluate human exposure to mycotoxins in buildings. The final phase of my project was to determine the presence of trichothecene mycotoxins in sera of individuals known to be exposed to *S. chartarum* in indoor environments. Combined with the known toxicity data of these compounds, the three phases of my research show a strong relationship between the

presence of toxigenic strains of *S. chartarum* and adverse human health effects in water-damaged "sick" buildings.

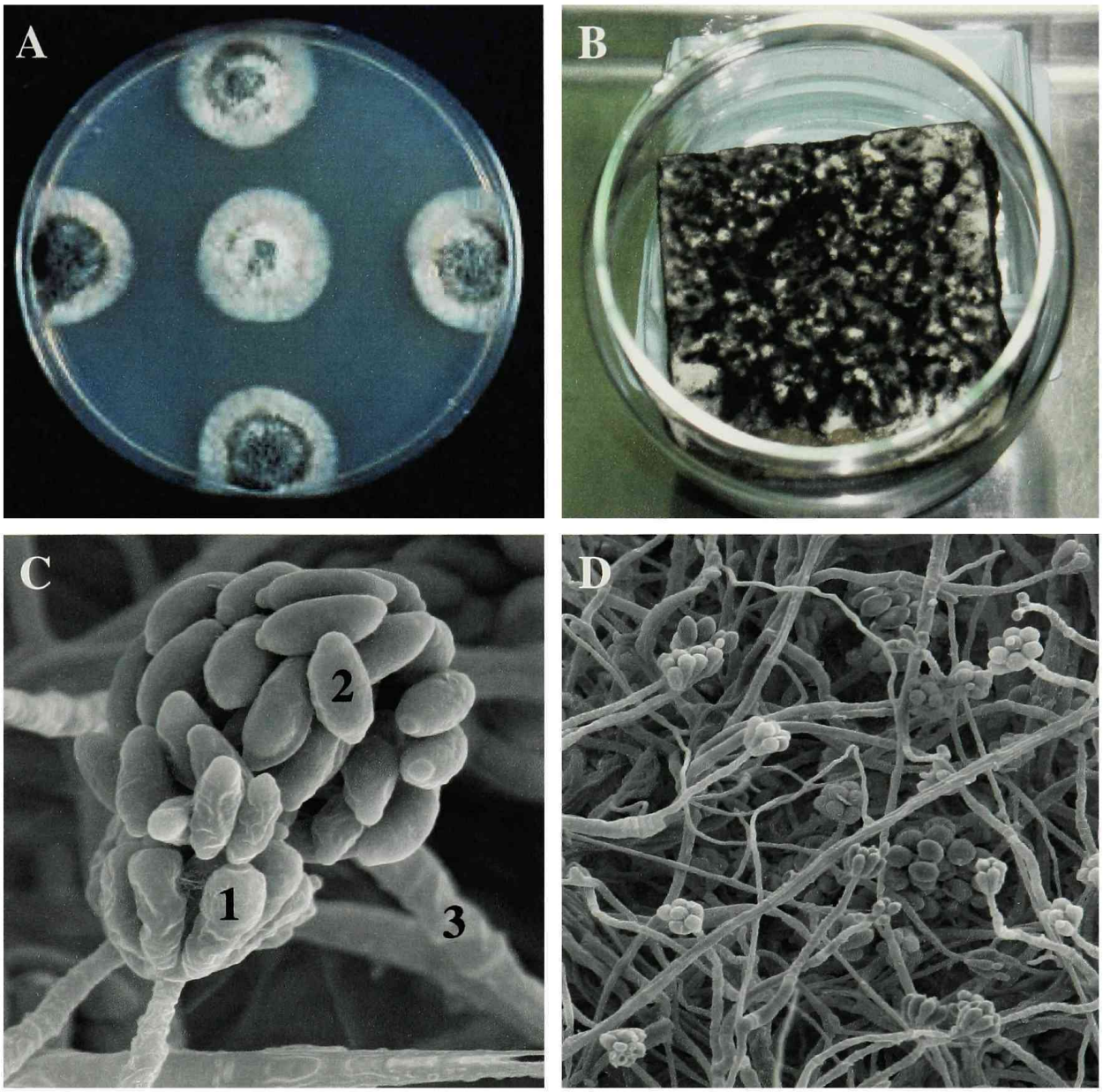

Figure 1.1. *Stachybotrys chartarum* Images. *A)* Seven-day-old *S. chartarum* colonies on potato dextrose agar *B)* Fourteen-day-old *S. chartarum* growth on cellulose-containing ceiling tile *C)* Scanning electron micrograph (SEM) of *S. chartarum* fruiting structure (conidiophore), conidia, and hyphae (1-3, respectively; magnification x800) *D)* SEM of *S. chartarum* hyphael mass with numerous fruiting structures and conidia (x160). [Electron microscopy performed by Dr. Dennis Kunkel, Dennis Kunkel Microscopy, Inc.]

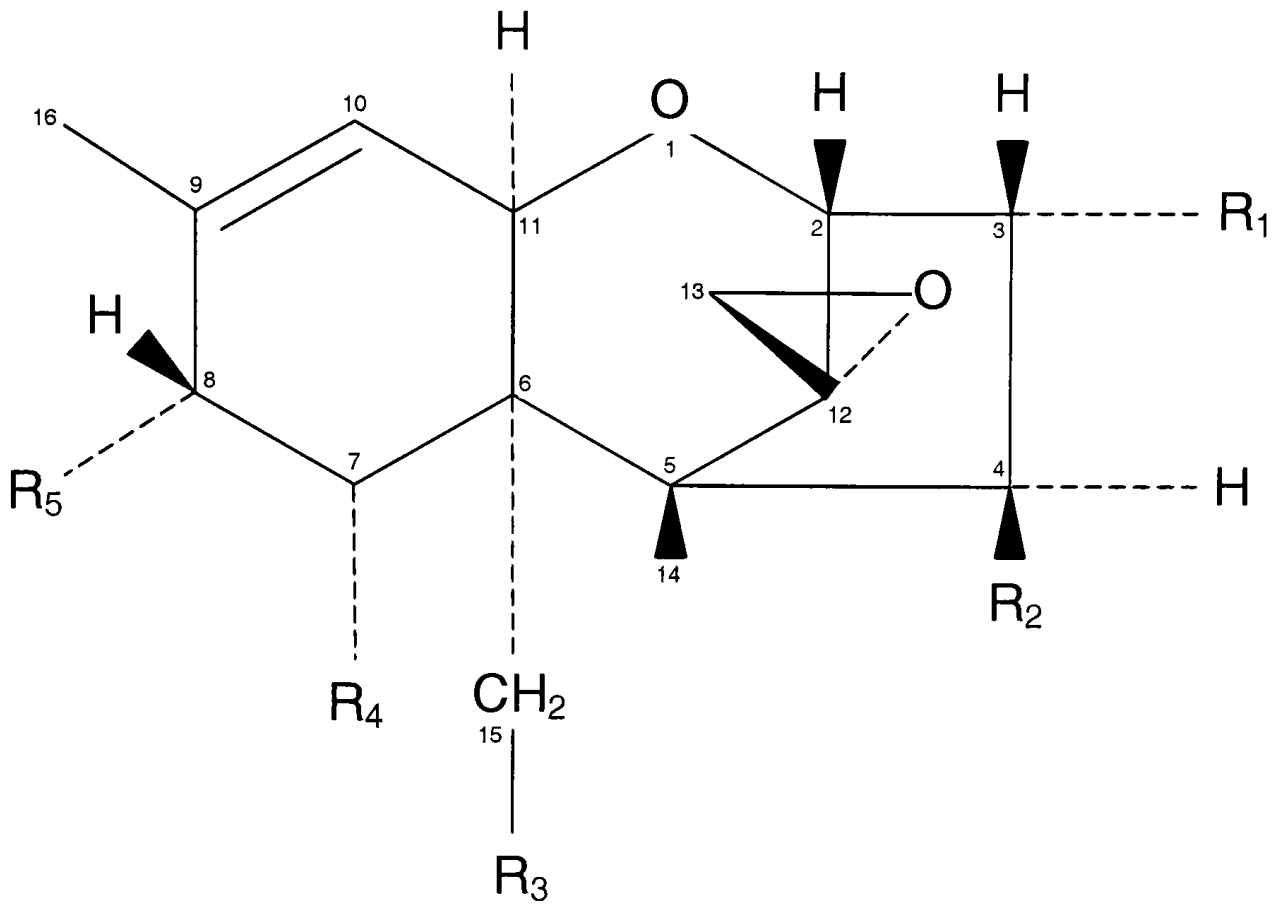

Figure 1.2. Basic Trichothecene Structure. Trichothecenes are classified based on difference in functional groups (R_1-R_5).

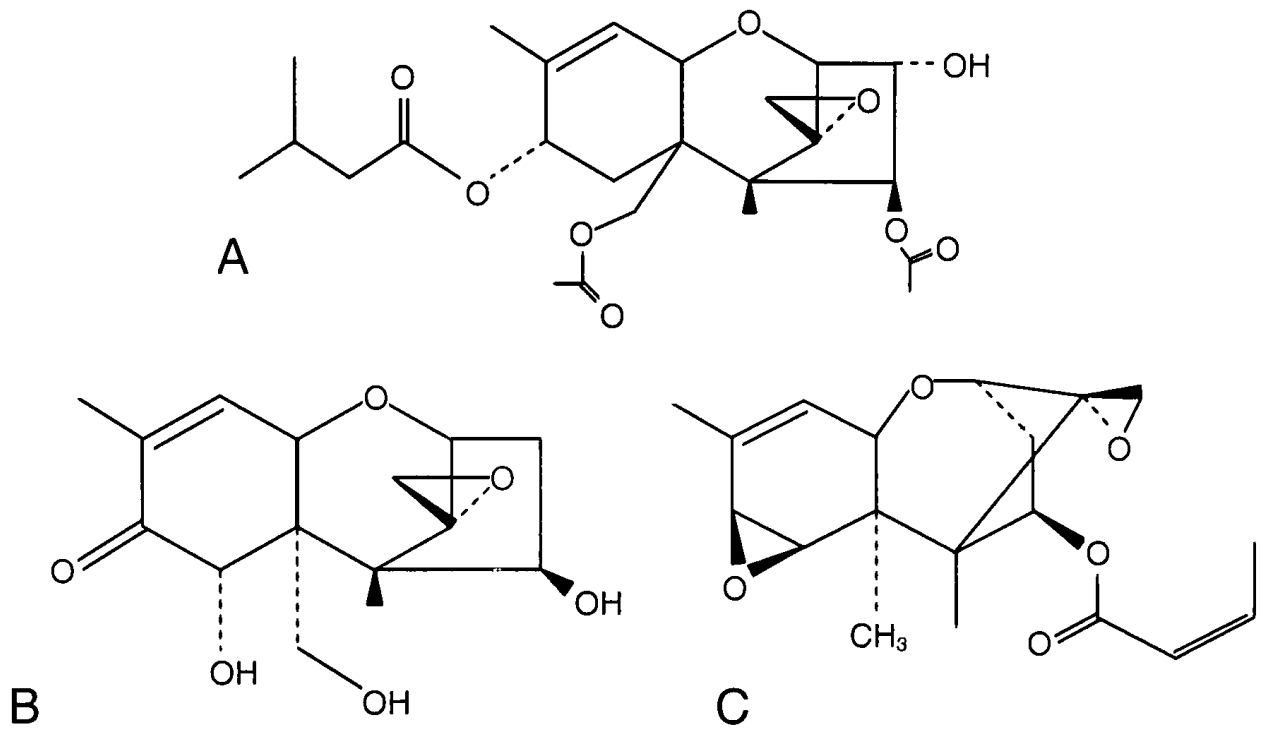

Figure 1.3. Trichothecene Classification System (Types A-C). *A)* T-2 toxin example of a Type A trichothecene mycotoxin *B)* Verrucarol – example of a Type B trichothecene mycotoxin *C)* Crotocin – example of a Type C trichothecene mycotoxin

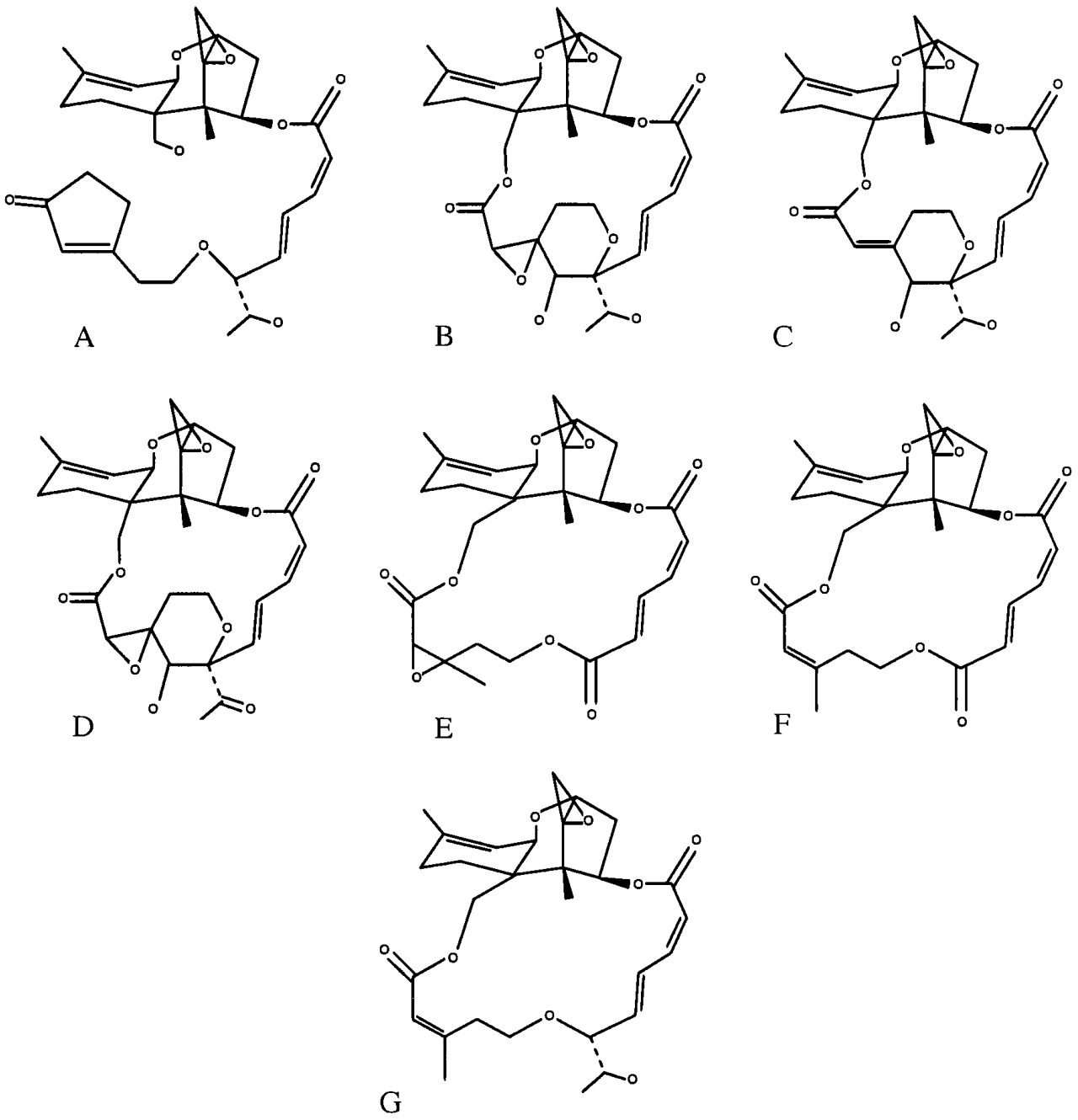

Figure 1.4. Select Type D (Macrocyclic) Trichothecene Mycotoxins Produced by *Stachybotrys chartarum.* A-G, respectively: roridin L-2, satratoxin G, satratoxin H, isosatratoxin F, verrucarin B, verrucarin J, roridin E

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CHAPTER II

DETECTION OF AIRBORNE *Stachybotrys chartarum* MACROCYCLIC TRICHOTHECENE MYCOTOXINS ON PARTICULATES SMALLER THAN CONIDIA

Introduction

The World Health Organization made the first attempt to define Sick Building Syndrome (SBS) in 1982. SBS has proven difficult to define and no single cause has ever been identified (2, 35). Complaints associated with poor indoor air quality range in severity and include difficulty in breathing, headaches, watering of the eyes, and flu-like symptoms, but are not limited to these (36). Numerous research groups have tried to determine the underlying cause(s) of SBS and poor indoor air quality (IAQ). Fungi and their secondary metabolites, such as mycotoxins, are hypothesized contributors that have been closely examined (3, 5, 6, 9, 18, 22). Fungi isolated from buildings with poor IAQ include a wide variety of genera and species. Recent research has shown that, along with airborne conidia, highly respirable fungal fragments can lead to human exposures in that the fragments can be aerosolized simultaneously with conidia. These amounts could be as high as 320 times that of conidia (16). Kildesø et al., (30) claimed that for a typical spore size (3 μm and density at 1 g/cm^3) the average mass exposure to spores may be approximately 0.14 μg/m^3. Expanding on their previous studies which concluded that the average concentration of respirable particles in a typical Danish office building was approximately 50 μg/m^3 of air (29), conidia would only be a small fraction of the potential particulates, possibly less than 1%, in contaminated buildings. This suggests that fungal fragments and other small particulates, such as dust and non-organic debris,

could be potential carriers for the majority of aerosolized mycotoxins, and therefore cause for concern and further study. A number of different fungi, including *Stachybotrys chartarum*, have been hypothesized to be important contributors to problems such as adverse human health effects associated with indoor fungal growth (6, 15, 38). *S. chartarum* has been proposed to be associated with human adverse health effects on a limited scale (8, 12, 13, 23, 24). Numerous compounds have been characterized from *S. chartarum.* It can produce anticomplement compounds and the immunosuppressive phenylspirodrimanes (31). Recently, a novel hemolysin named Stachylysin has been described (43). A group of compounds known as atranones has also been recently described (20). *S. chartarum* is also known to produce the cyclosporins, spirolactams, spirolactones (25), and spirocyclic drimanes (32) which are thought to possess immunosuppressive qualities. None of these, however, have been a main focus of study for ailments arising from exposure to *S. chartarum* in the indoor air. Instead, much interest has revolved around the trichothecene mycotoxins that *S. chartarum* produces (1, 28, 33, 34, 40, 41). These include, but are not limited to, the macrocyclic trichothecenes verrucarins B and J, roridin E, satratoxins F, G, and H, isosatratoxins F, G, and H and the non-macrocyclic trichoverrols and trichoverrins (19, 26). Recently, satratoxin G was found to be localized primarily in the conidia followed by other fungal constituents such as the phialides and hyphae (17). Several of these mycotoxins are known to react primarily with mucous membranes of the upper respiratory tract and eyes, leading to irritating erythema, inflammation, and pain (10). Inhalational studies in animals have shown the respiratory route of exposure to trichothecene mycotoxins is highly effective (7).

While the consequences of exposure to mycotoxins in buildings with poor IAQ are essentially unexplored (37), there is a substantial body of case studies and some laboratory evidence to suggest that they may contribute to reported complaints such as headaches, eye and throat irritation, nausea, dizziness, nose bleeds, and both physical and mental fatigue in subjects occupying such interiors (11, 37, 42). The members of the macrocyclic trichothecene family of mycotoxins are known inhibitors of protein synthesis in eukaryotes (14, 35, 44). Some studies hypothesize that they may also play a role in neurotoxicity and could therefore be particularly detrimental to humans (27). In light of the potential consequences of airborne mycotoxins on human health, it is important to know the possible ways in which mycotoxins can become airborne. For this study, we hypothesized that airborne *Stachybotrys chartarum* trichothecene mycotoxins (with a focus on macrocyclic trichothecenes) can potentially exist on particulates smaller than conidia (i.e., free from spores). The significance of this study lies in the fact that the majority of particulate matter found in indoor environments is highly respirable (significantly smaller than *S. chartarum* conidia) and that such particles are possible carriers of trichothecene mycotoxins.

Materials and Methods

Fungal Growth

A mycotoxin producing strain of *S. chartarum* (ATCC 201212) was selected for these experiments. The strain used has previously been shown to produce macrocyclic trichothecenes by high performance liquid chromatography (HPLC) analysis (25, 26). Stock cultures were maintained on sterile 7 X 7 cm cellulose ceiling tiles in a 25°C

incubator. To grow the organism, ceiling tile squares were first allowed to absorb 50 mi of autoclaved pyrogen-free water in sterile 100 mm x 80 mm Pyrex glass jars. One milliliter of *S. chartarum* conidia solution at a concentration of 1 x 10^6 conidia per milliliter of phosphate buffered saline (PBS) was evenly dispersed over the entire surface area of each piece of water-saturated ceiling tile. Conidia were collected from confluent growth cultures in 20 ml of PBS at pH 7.4. Collection was performed using sterile 25 ml disposable serological pipets (Fisher Scientific, Hampton, NH). Briefly, the cultures were washed with the PBS by repeated gentle aspirations (approximately 20 times or until the majority of the visible growth was removed). The conidia were then counted using a hemacytometer and diluted to the concentration mentioned above. This working solution was used for the immediate inoculation of ceiling tiles used in the present experiments. Similar for stock cultures, ceiling tiles were considered ready for air sampling experiments after confluent growth was reached (fourteen days).

Air Sampling and Particulate Collection

These experiments were run a total of 12 times (including controls) under various sampling times (mentioned below). The air sampling apparatus for collecting macrocyclic trichothecenes on particulates is depicted in Figure 2.1. The pieces of ceiling tile that contained confluent fourteen-day-old *S. chartarum* cultures were cut into one-inch squares using a sterile scalpel blade under a class II biological safety cabinet. The pieces were wet at the time of cutting (minimizing loss of conidia and mycelial fragments), but were quickly dried (due to the relatively high flow rate) as collection began. Gas-drying tubes (Drierite, Xenia, OH) were emptied of their supplied dessicant,

thoroughly cleaned with a soapy water/2% bleach solution, and dried before use. A total of 14 squares of ceiling tile were placed in each tube. A total of six tubes connected in series using Nalgene (Rochester, NY) polyvinyl chloride tubing were used for air sampling purposes. Incoming air was filtered using a Whatman (Clifton, NJ) EPM-2000 glass microfiber filter. EPM filter material was selected by the United States Environmental Protection Agency as the standard for use in high volume air sampling. According to the manufacturer, they are 99.99% efficient for 0.3 μm-sized dioctyl phthalate particles (standard particles for testing filter efficiency) at a 5 cm/sec flowrate. For this reason, they were chosen to ensure that clean, particulate-free air was entering the experimental apparatus. Prior to each sampling period, the sealed tubes were vigorously shaken by hand (25 times up and down). This was done similarly each time to initially generate intact conidia, mycelia and fragments from these fungal constituents. Air moving at a flow rate of 30 liters per minute (LPM), as measured by a flow meter (Gilmont Instruments, Barrington, IL) at the exit, was passed through the connected tubes with a negligible pressure drop throughout the apparatus. Particulates were collected on a series of membrane filters housed in 47 mm Fisherbrand® gas line low-pressure filter holders (Fisher Scientific) that were placed at the exit of the air sampling setup in order of decreasing pore size. For these experiments, Millipore (Billerica, MA) polycarbonate filters of pore sizes 5.0, 1.2, and 0.4 μm were used. Sampling times were 1, 3, 6, 12, 24, 48, and 72 hours. Additionally, for the collection of material used for HPLC analysis, the same procedure was followed, but for an extended sampling time of 120 hours. Tubes containing squares of sterile ceiling tile alone were used as controls. They were similarly

sampled for 1, 6, 12, and 24 hours for comparison. Each collection period was performed once.

Additional experiments were also performed in a similar manner using *S. chartarum*-contaminated rice rather than ceiling tile. The production of macrocyclic trichothecenes by *Stachybotrys* is greatly increased when rice is used as a substrate (21). However, when using this method, growth and extraction processes are extremely time-consuming and thus the reason why ceiling tile was chosen as a substrate for the majority of the experiments. Additionally, ceiling tile is a more natural substrate (in regards to contaminated indoor environments) than rice. With these added trials, we were also able vary the filter pore sizes and experimental flow rates and shorten the sampling times with similar results. Descriptions and data for these experiments can be found in Appendix B.

Scanning Electron Microscopy

One-quarter of the area (approximately 435 mm^2) was cut from the test filters using a sterile scalpel blade immediately following 72 hour air sampling. This was done in duplicate to retrieve the best quality images for SEM. The remaining portion of each filter was used for ELISA and HPLC confirmation as already described. The pieces for SEM were individually placed in clean, sterile 20 ml scintillation vials. Samples were mounted on studs and gold-coated. After coating, samples were kept at 0°C in the gold coater until scanning, so as to prevent any outside contamination. They were scanned using a Hitachi S-500 scanning electron microscope (Hitachi America, Ltd. San Francisco, CA).

Sample Extraction and Preparation

Following air sampling, filters were removed from the filter holders and examined macroscopically for any tears or large holes. If none were seen, the filters were then individually placed in 20 ml scintillation vials. Each was submerged in 15 ml of HPLC-grade methanol. The vials were vortexed for 60 seconds and the filters were immediately removed with sterile forceps. The filter extracts were evaporated to dryness using the TurboVap II Concentration workstation (Zymark Corporation, Hopkinton, MA). Dried extracts were resuspended in 1 ml of 5% HPLC-grade methanol in PBS and filtered through 13 mm nylon syringe filters with a 0.22 μm pore size (Millipore). Filtering the samples in this manner had a negligible effect on trichothecene concentrations. This was the final working sample for the enzyme-linked immunosorbent assay (ELISA). For comparison purposes, sterile, unexposed filters were treated in the same manner.

Macrocyclic Trichothecene Mycotoxin Detection

Macrocyclic trichothecenes were detected using the QuantiTox Kit for trichothecenes (EnviroLogix, Portland, ME). This competitive ELISA kit incorporates trichothecene-specific antibodies developed and previously described by Chung et al., (4) immobilized on polystyrene microtiter wells. All reagents and antibody-coated wells were allowed to equilibrate to room temperature before use. Briefly, 170 μl of filter extract was mixed with 170 μl of horseradish peroxidase (HRP)-conjugated satratoxin G in separate 1.5 ml centrifuge tubes. The tubes were vortexed to ensure proper mixing. For testing, 100 μl of this sample or control mixture was added to wells in triplicate. Wells were covered and incubated at room temperature on a plate rocker for 45 minutes.

Following incubation, wells were washed five times with PBS at pH 7.4 using the Dynatech MRW plate washer (Chantilly, VA). Wells were blotted dry on clean paper towels. Immediately, 100 µl of tetramethylbenzidine substrate solution was added to each well. This was allowed to incubate at room temperature under reduced lighting for 15 minutes. To stop the reaction, 100 µl of 1N hydrochloric acid (stop solution) was added to each well. Wells were read at 450 nm using the EL-312 microtiter plate reader (Bio-Tek Instruments, Winooski, VT). Inhibitions were based on samples versus appropriate controls and represent the degree of inhibition the test sample had on the capability of the satratoxin G-HRP conjugate to bind to the immobilized antibody. Cross-reactivities to other trichothecenes and two non-trichothecene mycotoxins were investigated to confirm the efficacy of the ELISA. All toxins were diluted to the same concentrations (50 ng/ml, 5 ng/ml, 500 pg/ml, and 50 pg/ml) in PBS containing 5% (v/v) HPLC-grade methanol and tested in triplicate wells. Satratoxins G and H were purified as described by Hinkley et al., (19) in our laboratory. Roridin A, verrucarin A, deoxynivalenol, verrucarol, diacetylverrucarol, neosolaniol, and T-2 toxin were purchased from Sigma (St. Louis, MO). Altenuene (Sigma) and sterigmatocystin (Acros Organics, Belgium), non-trichothecene mycotoxins, were also tested. Roridin A, at a concentration of 1 µg/ml, was used as a positive control for each ELISA performed.

ELISA Interpretation and Statistical Analysis

Percent inhibitions were calculated as done by Schick et al. (39) using the formula:

% Inhibition = 100 x 1 - [(O.D.$_{450}$ sample – background)/(O.D.$_{450}$ control-background)]

Statistical analyses were performed using Sigma Stat 2.0 software (Chicago, IL). Toxin concentrations were compared to solvent alone (5% methanol in PBS) and individually compared using a Student's *t*-test ($P<0.05$). Filter extracts were compared to controls (similar unused filters) using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis ($P<0.05$). All requirements for normality were met before running these analyses.

For the filter extracts, data are also presented as relative concentrations of macrocyclic trichothecene mycotoxins. This was done using an ELISA-based macrocyclic trichothecene standard curve. The standard curve was developed by testing a mixture of four macrocyclic trichothecenes (satratoxins G and H, verrucarin A, and roridin A) in equal amounts via the ELISA as already described. Dilutions were made in PBS from a concentrated stock solution of the toxins in methanol (250 μg/ml of each toxin) resulting in 12 test concentrations: 500, 250, 100, 50, 25, 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 ng/ml. Average ELISA absorbances (from three replicates) at 450 nanometers were then plotted against toxin concentrations (based on dilutions) to generate a standard curve. Statistical analyses were the same as described for percent inhibition comparisons.

HPLC Analysis

HPLC analysis was performed using an Agilent Technologies 1100 Series HPLC System (Palo Alto, CA) equipped with an ultraviolet-visible diode array detector. A 400 mm (250 mm + 150 mm) x 4.6 mm Eclipse C8, 5 micron particle size analytical column

plus a 12.5 mm guard column set at 40°C was used for the analyses. The flow rate was set at 1.0 ml/min with an injection volume of 10 µl for purified satratoxin H and 100 µl for the filter extracts. Filter extract samples (as described for ELISA analysis) were run in a mobile phase in which the gradient changed from 35% of 5% acetonitrile in water to 70% acetonitrile in 14 minutes. Samples were read at 260 nm and analyzed using Chemstation software (Agilent). The method was quantitatively calibrated for satratoxin H.

Results

Scanning Electron Microscopy

SEM demonstrated successful particle separation and collection through our air sampling apparatus. *S. chartarum* conidia were clearly identified on the 5.0 µm pore size filters (Figure 2.2A) that we hypothesized to capture most conidia. However, we did not rule out the possibility that conidia could still pass through this pore size. Figure 2.2B (arrow) shows a *S. chartarum* conidium oriented perpendicularly to the filter and lodged in a pore. This implies that some conidia could have passed through to the next filter in series. However, from our observations, this was not the case. We did not find intact *S. chartarum* conidia on the 1.2 µm pore size filters, but rather various particulates and other debris (Figure 2.2C). Figures 2.2D and 2.2E show that we also captured extremely small particles (<0.5 µm based on the scale) on the 0.4 µm pore size filters (arrows).

ELISA Cross-reactivity Characterization

As Table 2.1 shows, the QuantiTox ELISA kit proved suitable for detection of macrocyclic trichothecenes at several dilutions. No toxins showed statistically significant competitive inhibition at a concentration of 50 pg/ml. Significant values were defined as having a P-value less than or equal to 0.05 using the Student's *t*-test. Significant values for satratoxin G were seen at 50 and 5 ng/ml but not at 500 pg/ml. Significant inhibitions were seen for satratoxin H, roridin A, and verrucarin A at 50 ng/ml, 5 ng/ml, and 500 pg/ml. Verrucarol, a non-macrocyclic trichothecene mycotoxin demonstrated competitive inhibition at 50 and 5 ng/ml, but did not demonstrate the same high inhibitions as the macrocyclic trichothecenes. Diacetylverrucarol, a derivative of verrucarol, exhibited relatively high binding only at 50 and 5 ng/ml. At 500 pg/ml, the average inhibition for neosolaniol was significant, but a large standard deviation (± 12.6%) was observed at this concentration. All other tested toxins were negative or relatively unreactive.

Airborne Macrocyclic Trichothecene Mycotoxin Detection

The existence of airborne macrocyclic trichothecenes was confirmed using our filter apparatus on *S. chartarum* conidia, equivalent size particulates, and smaller particles. Values determined to be statistically significant (versus solvent alone) had a P-value of less than or equal to 0.05 using a one-way analysis of variance. Table 2.2 shows competitive inhibition percentages and semi-quantitative macrocyclic trichothecene estimates of the polycarbonate filter extracts. The trichothecene equivalents were determined using the standard curve shown in Figure 2.3. As can be seen, all inhibition

rates were high with the first filter (5 μm pore size) extracts averaging 94.5%. Average trichothecene equivalents were equally high averaging over 500 ng/ml. The second filter (1.2 μm pore size) extracts also demonstrated high inhibition and toxin concentrations (averaging 89.3% and 260.2 ng/ml, respectively). Overall, the 48-hour sample contained lower amounts of toxin. The third filters (0.4 μm pore size) collected extremely small (<0.5 μm) conidia-free particulates as demonstrated by SEM. Extracts showed significant reactivity in the ELISA, although not as high as the 5 and 1.2 μm pore size filters. The inhibition percentages averaged 45.1% and the trichothecene equivalents averaged 1.1 ng/ml with a general trend of increasing toxicity as sampling time increased. Again, the 48-hour sample contained significantly lower macrocyclic trichothecene content. Extracts from filters used for sampling ceiling tile alone showed moderate binding that increased with time (specifically on the 5 μm pore size filters). However, percent inhibitions were much lower than extracts from filters when *S. chartarum* was used.

HPLC Analysis

Figure 2.4 shows chromatograms of extracts from a 120-hour sampling. A longer sampling time was chosen because the other time points (as described in Table 2.2) did not contain sufficient amounts of sample for HPLC analysis [data not shown]. Figure 2.4A is a satratoxin H standard at a concentration of 1 ng/μl. UV spectrum analysis confirmed the presence of satratoxin H. Figure 2.4B shows the chromatogram of the filter extract from the 5.0 μm filter, clearly demonstrating the presence of satratoxin H

(inset). The retention time of 9.842 minutes is slightly different from the standard shown in Figure 4A due to the larger injection volume. Figure 2.4C represents the chromatogram of the 1.2 μm filter extract. Although there is no clear indication of satratoxin H, there is a major peak at 9.845 minutes where satratoxin H was demonstrated on the 5.0 μm filter. The UV spectrum of this peak (inset) shares similar qualities with satratoxin H (major absorbances at approximately 235 and 265 nm). Figure 2.4D shows the chromatogram of the extract from the 0.4 μm filter. As with the 1.2 μm filter, a satratoxin-like peak eluted at 9.848 minutes.

Discussion

In this study, we were successful in demonstrating the presence of airborne *Stachybotrys chartarum* macrocyclic (and possibly non-macrocyclic) trichothecenes on filters in the absence of conidia. This finding has implications for indoor air quality investigations involving *S. chartarum* growth on building material and the ensuing occupant complaints in that individuals could potentially be exposed to these potent toxins for extended periods of time via the respiratory route. Using a controlled air flow system incorporating *S. chartarum* growth on cellulose ceiling tile, we were able to demonstrate airborne macrocyclic trichothecenes on smaller than conidia size particles. The initial mechanical generation of particles via shaking was a means to shorten the overall sampling times for these experiments. Such intense disturbance and consequent release of particulates inclusive of *S. chartarum* conidia would not be expected to occur in native environments. However, subtler disturbance mechanisms (human and mechanical vibrations, fans, air conditioning units, etc.) are hypothesized to cause the

persistent release of such particulate matter over a longer period of time. The SEM images demonstrated that most of the intact conidia were captured on the 5.0 μm filters with very few, if any, seen on the 1.2 μm filters. SEM clearly showed particles 0.5 μm and smaller in diameter on the 0.4 μm filters after 72 hours of sampling (Figure 2.2D and 2.2E). It was unclear whether these were fungal fragments, pieces of substrate, or other debris. In addition, particles of this size were difficult to locate and observe on the final filter. However, this does not mean that they existed in low quantity. The fact that we observed very low numbers may simply be due to the mechanical capture of the bulk of the smaller particles on the first filter(s). To further emphasize this phenomenon, our laboratory conducted similar tests (Appendix B) using larger pore size (up to 20 μm) membrane filters. Our results showed that the majority of *Stachybotrys* conidia were captured on these filters even though the pores were easily large enough to allow conidia passage. We believe this phenomenon occurred on the 5 and 1.2 μm pore size polycarbonate filters and that very few extremely minute particulates (carrying toxin) passed through to the final filter.

We were able to show that an ELISA incorporating a macrocyclic trichothecene mycotoxin-specific antibody can be used for determining airborne concentrations of these toxins. Our results correlate with data recently published by Chung et al., (4) thereby attesting to the efficacy of the ELISA. It was not surprising to find cross-reactivities with non-macrocyclic trichothecenes. All trichothecene mycotoxins share a central trichothecene moiety. The antibody used for our detection purposes was raised against a low molecular weight compound (satratoxin G). When compared to an antibody against a very specific tertiary structure (such as a protein) it is expected that a small degree of

cross-reactivity/non-specific binding would occur. Because of this, other compounds with similar structures, such as other mycotoxins, may result in a background noise. Nonetheless, we are confident that the ELISA used here is highly specific for macrocyclic trichothecenes.

Filter extracts demonstrated significant inhibition proportionate to the length of sampling time. Extracts from 48-hour sampling skewed our results and the reasoning for this is uncertain. One possible explanation is that the shaking process could have resulted in uneven disturbance for this particular trial. Nonetheless, we were still able to demonstrate the presence of mycotoxins. We also demonstrated that filter extracts from sampling using sterile ceiling tile alone showed significant ELISA reactivity. This could be attributed to the sensitivity of the ELISA and may have falsely increased our values. The high concentration of particles initially generated by hand could have overwhelmed the test (essentially resulting in a high background noise). We do not believe that trichothecenes were present on the sterile ceiling tile. If they were originally present (due to cross-contamination, for instance) they would have been destroyed through the autoclaving used to sterilize the ceiling tile (45). To further strengthen our conclusions, we performed similar air collection experiments using *Stachybotrys*-contaminated rice (Appendix B). Sterile rice alone did not result in false positives [data not shown]. However, the particulate/trichothecene separation was still successful. The combined data obtained from the filter experiments demonstrated that macrocyclic trichothecenes were found without captured intact conidia. Correlating to standards of similar toxins, our filter extracts contained 500 parts per trillion to greater than 50 parts per billion of macrocyclic trichothecenes. We also demonstrated the presence of satratoxin H (through

HPLC analysis) associated with conidia, but only speculatively with smaller size particulates such as fungal fragments. We were able to identify peaks eluting at the same retention time as seen for satratoxin H on the 5.0 μm filter that were similar in nature, but not definitively satratoxin H. This lack of certainty is most likely due to the significantly lower amount of satratoxin H present on the 1.2 μm filter, as demonstrated by a 10-fold decrease in milli-absorbance units (mAUs), in combination with the large injection volume and limit of detection. For the HPLC methods and equipment used in these experiments, the limit of detection was approximately 10 pg/μl when injecting a large volume (100 μl) of purified toxin. This indicates that the ELISA technology used in our experiments was more sensitive and/or specific than the applied HPLC methods (100 pg/ml versus 10 pg/μl). This is important in that the levels of trichothecenes from *S. chartarum* in contaminated indoor environments are expected to be very low at any given time and a sensitive means of detection is necessary. To date however, no data exist describing what airborne concentrations of these toxins are necessary to adversely effect human health.

Most indoor air quality investigations focus on surface growth and airborne conidia concentrations. As Górny et al. concluded, spore counts do not adequately represent the amount of fungal fragments that are present in the air at any given time (16). In fact, fragments and equal size particles greatly outnumber intact fungal spores. Based on our results, we feel that air sampling in *S. chartarum*-contaminated indoor environments should incorporate a means of collecting particulates smaller than conidia followed by a specific and sensitive test for mycotoxins. For example, collection and separation could be done by using a filtration setup similar to what we have presented

here. Also, current cyclone technology that allows for the exclusion of certain size particles (such as conidia) could be used in conjunction with the numerous types of air sampling devices that are available. Digital particle counters/analyzers would also be ideal for characterizing and enumerating the collected particles. Finally, it should be noted that background "normal" levels of such toxins, particularly as determined using the ELISA presented here, have yet to be determined.

Table 2.1. Competitive ELISA inhibition of select trichothecene and two non-trichothecene mycotoxins.

Toxin[a]	Concentration[b] and Average % Inhibition[c]			
	50 ng/ml	*5 ng/ml*	*500 pg/ml*	*50 pg/ml*
Satratoxin G	58.4 ± 2.85*	24.4 ± 1.75*	9.23 ± 6.14	0.00**
Satratoxin H	79.3 ± 1.56*	62.5 ± 0.62*	28.2 ± 2.24*	0.00**
Roridin A	83.5 ± 0.51*	59.8 ± 5.16*	43.3 ± 10.1*	0.00**
Verrucarin A	78.6 ± 1.82*	51.5 ± 1.60*	14.2 ± 2.22*	0.00**
Deoxynivalenol	0.195 ± 3.33	0.00**	0.038 ± 2.59	0.00**
T-2 Toxin	1.19 ± 2.06	0.00**	0.00**	0.00**
Verrucarol	18.2 ± 5.65*	11.1 ± 2.95*	3.40 ± 2.22	0.00**
Diacetyverrucarol	51.7 ± 4.11*	25.3 ± 9.20*	12.1 ± 4.82*	0.00**
Neosolaniol	5.43 ± 4.08	6.96 ± 3.47	20.5 ± 12.6*	6.64 ± 2.72
Altenuene	0.00**	0.00**	0.00**	0.00**
Sterigmatocystin	0.00**	0.00**	8.49 ± 1.01	0.00**

[a]Satratoxins G and H, roridin A, and verrucarin A are macrocyclic trichothecene mycotoxins. Deoxynivalenol, T-2 toxin, verrucarol, diacetylverrucarol and neosolaniol are non-macrocyclic trichothecene mycotoxins. Altenuene and sterigmatocystin are non-trichothecene mycotoxins.

[b]Dilutions were made in 5% methanol in PBS

[c]Results are based on solvent-only (5% methanol in PBS) controls. Values represent triplicate wells. Standard deviations are shown. Values significant as determined by a Student's *t*-test ($P<0.05$) are noted with an *. All negative inhibition values were converted to 0.00% inhibition and are noted with a **.

Table 2.2. Competitive ELISA inhibition and trichothecene equivalents of polycarbonate filter extracts – *Stachybotrys*-contaminated ceiling tile setup.

Filter pore size[a]	Sampling time[b]	Average % Inhibition[c]	Average Trichothecene Equivalent[d]
5.0		96.17 ± 0.08*	> 500*
1.2	1	94.82 ± 0.08*	335.96 ± 13.09*
0.4		52.50 ± 1.00*	1.00 ± 0.06*
5.0		96.08 ± 0.20*	> 500*
1.2	3	94.57 ± 0.24*	298.72 ± 33.95*
0.4		37.24 ± 3.03*	0.48 ± 0.06*
5.0		95.99 ± 0.16*	> 500*
1.2	6	94.88 ± 0.14*	348.34 ± 24.65*
0.4		38.36 ± 0.32*	0.50 ± 0.01*
5.0		96.42 ± 0.14*	> 500*
1.2	12	95.45 ± 0.14*	473.71 ± 39.42*
0.4		46.61 ± 4.19*	0.75 ± 0.17*
5.0		96.30 ± 0.26*	> 500*
1.2	24	94.50 ± 0.28*	289.75 ± 37.42*
0.4		71.93 ± 1.18*	3.99 ± 0.42*
5.0		85.28 ± 0.66*	21.76 ± 2.62*
1.2	48	60.16 ± 3.23*	1.61 ± 0.33*
0.4		17.35 ± 4.90*	0.23 ± 0.04*

Table 2.2. Continued.

Filter pore size[a]	Sampling time[b]	Average % Inhibition[c]	Average Trichothecene Equivalent[d]
5.0		95.13 ± 0.36*	402.48 ± 72.68*
1.2	72	90.71 ± 0.45*	73.05 ± 8.84*
0.4		51.57 ± 0.75*	0.95 ± 0.04*
5.0†		15.71 ± 1.82*	0.22 ± 0.01*
1.2†	1	11.83 ± 3.01*	0.20 ± 0.02*
0.4†		3.44 ± 1.43	0.15 ± 0.01
5.0†		5.97 ± 1.61	0.17 ± 0.01
1.2†	6	12.08 ± 0.49*	0.20 ± 0.00*
0.4†		0.00**	0.14 ± 0.01
5.0†		32.07 ± 4.31*	0.39 ± 0.07*
1.2†	12	9.35 ± 1.88*	0.18 ± 0.01*
0.4†		18.50 ± 3.52*	0.24 ± 0.03*
5.0†		37.46 ± 2.56*	0.49 ± 0.05*
1.2†	24	15.75 ± 6.92*	0.23 ± 0.05*
0.4†		14.62 ± 1.85*	0.21 ± 0.01*

Table 2.2. Continued.

Filter pore size[a]	Sampling time[b]	Average % Inhibition[c]	Average Trichothecene Equivalent[d]
5.0††		0.00**	0.13 ± 0.02
1.2††	NA	0.00**	0.14 ± 0.01
0.4††		0.00**	0.12 ± 0.01

[a]In μm. Filters are grouped based on order of the series and sampling time. Samples indicated with a † represent those tests using sterile ceiling only. Samples indicated with a †† represent extracts of sterile filters alone.

[b]In hours. Each sampling period was performed one time. NA; not applicable.

[c]Results are based on solvent-only (5% methanol in PBS) controls. Values represent triplicate wells. Standard deviations are shown. Those values significant as determined by a one-way ANOVA ($P<0.05$) are noted with an *. Test groups (*Stachybotrys* on ceiling tile) at 1, 6, 12, and 24 hours were compared to correlating sampling times using ceiling tile alone and were significantly different ($P<0.05$). All negative inhibition values were converted to 0.00% and are noted with a **.

[d]In ng/ml. Values are semi-quantitative and are based on the macrocyclic trichothecene standard curve presented in Figure 2.3. Statistical analyses were performed as described for percent inhibition comparisons.

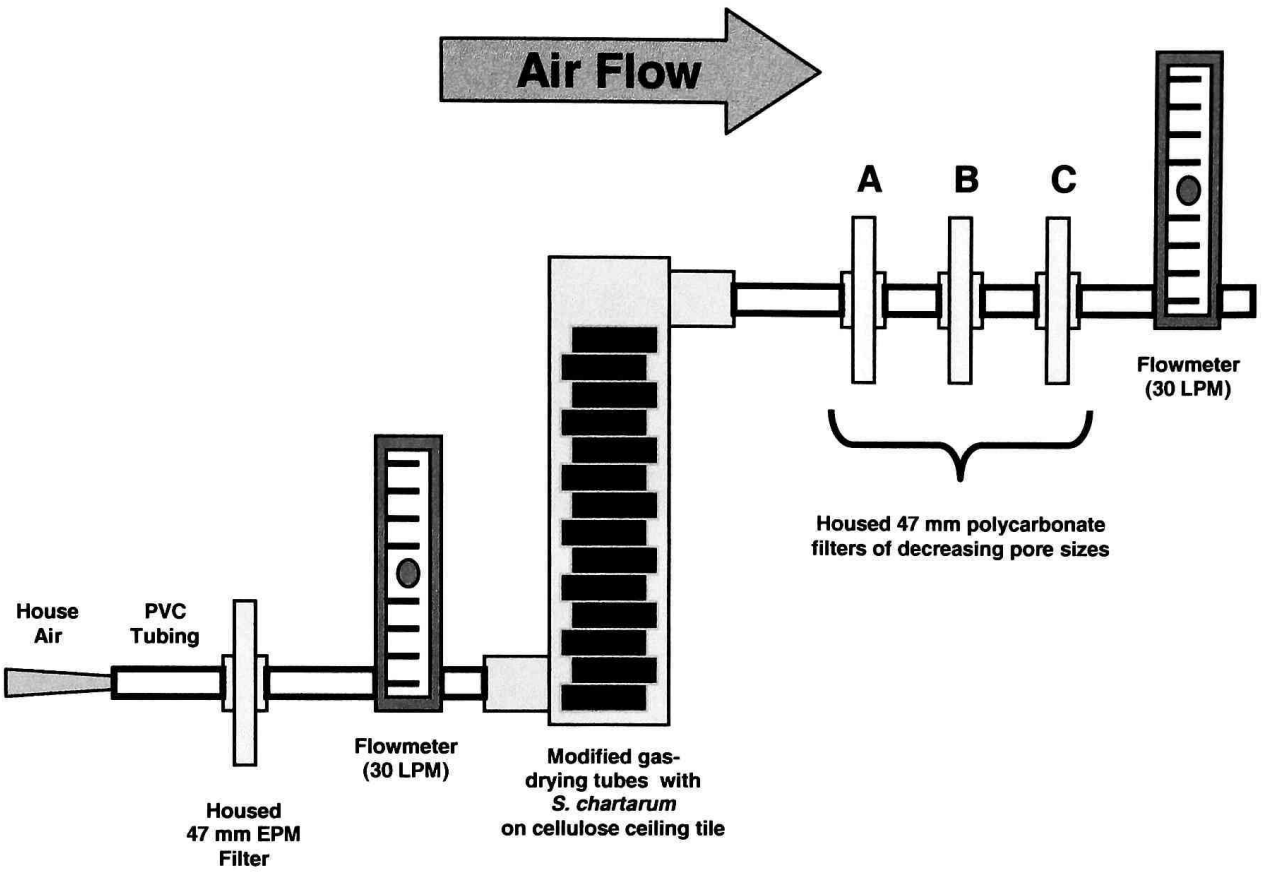


Figure 2.1. Experimental Air Sampling Apparatus. Filtered house air at 30 liters per minute (LPM) was passed over cellulose ceiling tile with confluent *Stachybotrys chartarum* growth for various periods of time. A total of six gas-drying tubes representing approximately 1,176 cm^2 of *Stachybotrys chartarum* growth were connected using polyvinyl chloride (PVC) tubing. Particles were separated and collected on 47 mm diameter polycarbonate membrane filters with pore sizes of (A) 5.0 μm, (B) 1.2 μm, and (C) 0.4 μm and later analyzed for presence of macrocyclic trichothecenes.

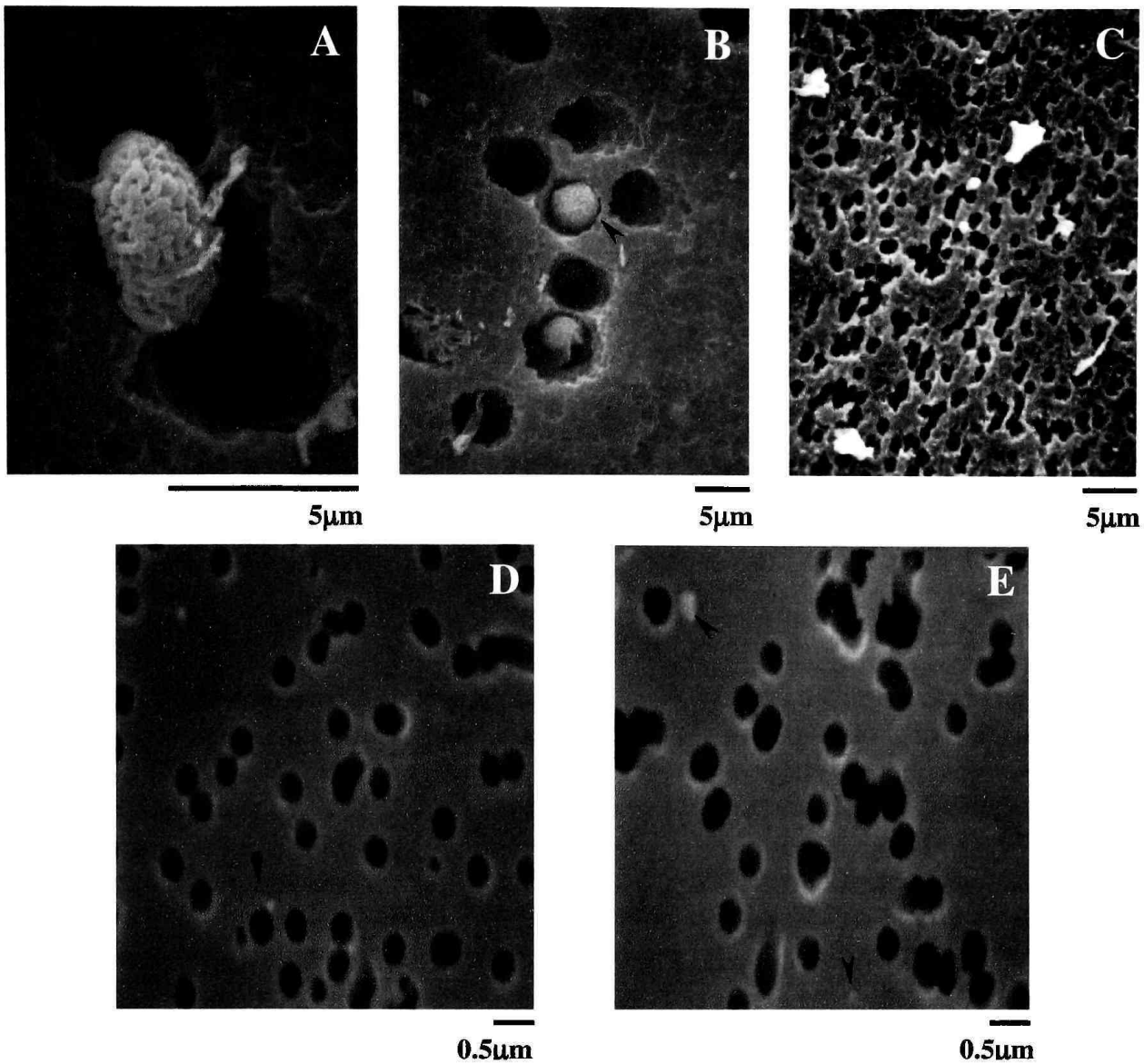
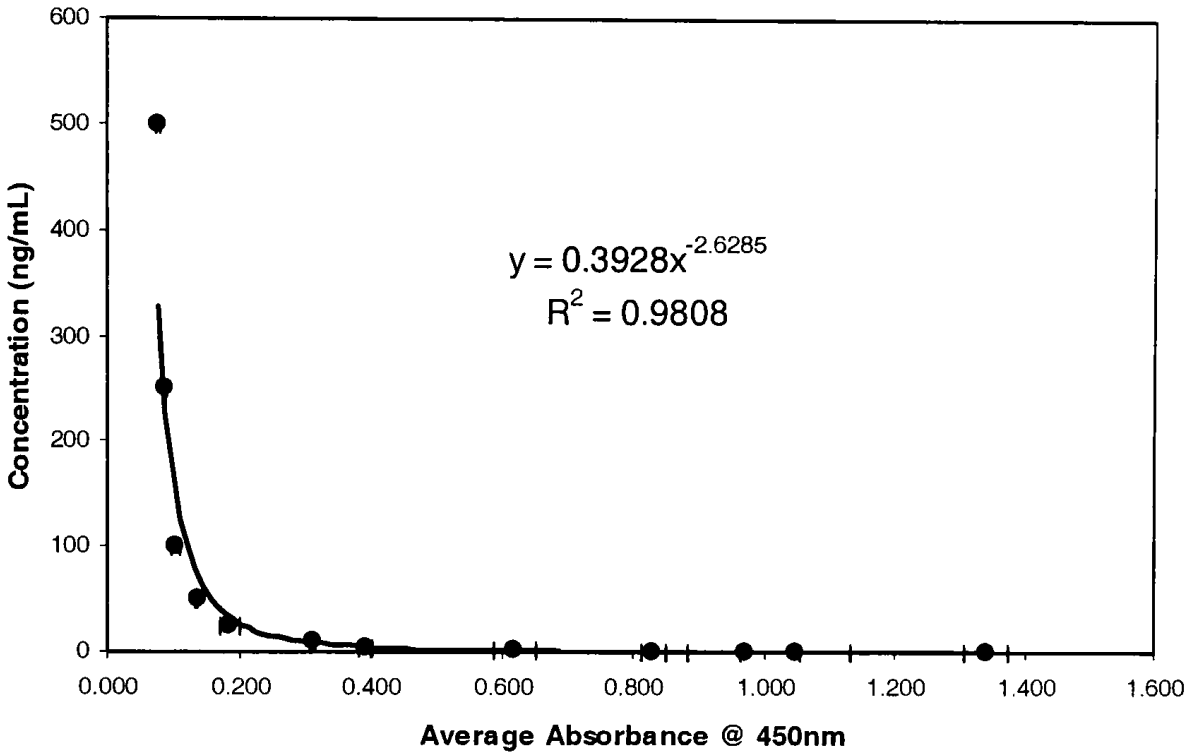


Figure 2.2. Scanning Electron Micrographs of Polycarbonate Membrane Filters Following 72 Hours of Sampling from Experimental Air Sampling Apparatus. The filter pores are clearly distinguishable from captured particulate matter (dark spherical versus irregularly shaped bodies, respectively). Filter (5.0 μm pore size) magnified 5000x demonstrating a captured *S. chartarum* spore (A). The same filter type magnified 2000x with an intact *S. chartarum* spore lodged in a pore (arrow) (B). Filter (1.2 μm pore size) magnified 2500x showing no *S. chartarum* spores but a significant amount of debris (C). Filters (0.4 μm pore size) at a magnification of 10,000 showing extremely small captured particulates (arrows) (D and E).

Figure 2.3. ELISA Based Macrocyclic Trichothecene Standard Curve. Satratoxins G and H, roridin A, and verrucarin A were mixed in equal concentrations in methanol, diluted in PBS (500 to 0.1 ng/ml), and tested using a macrocyclic trichothecene-specific ELISA. OD_{450}s were plotted against toxin concentrations as the power curve shown here. This standard curve was used to estimate macrocyclic trichothecene equivalents from experimental filter extracts. For reference, the trichothecene concentrations, OD_{450}s, and percent inhibitions are shown in a table format below the graph. Standard deviations (representing three replicates) for all values are noted on the graph and in the table.

Macrocyclic Trichothecene Standard Curve Using Roridin A, Verrucarin A, and Satratoxins G & H - Average Concentrations Based on ELISA (500 to 0.1 ng/mL)



Concentration (ng/ml)	Avg OD_{450}	Avg % Inhibition
500	0.08 ± 0.00	94.6 ± 0.2
250	0.09 ± 0.00	93.8 ± 0.3
100	0.11 ± 0.01	92.6 ± 0.5
50	0.14 ± 0.00	90.3 ± 0.1
25	0.19 ± 0.02	86.9 ± 1.1
10	0.31 ± 0.00	78.0 ± 0.2
5	0.39 ± 0.01	72.4 ± 0.7
2.5	0.62 ± 0.03	56.4 ± 2.2
1	0.83 ± 0.02	41.5 ± 1.3
0.5	0.97 ± 0.09	31.7 ± 6.1
0.25	1.05 ± 0.08	26.2 ± 5.8
0.1	1.34 ± 0.03	5.6 ± 2.4

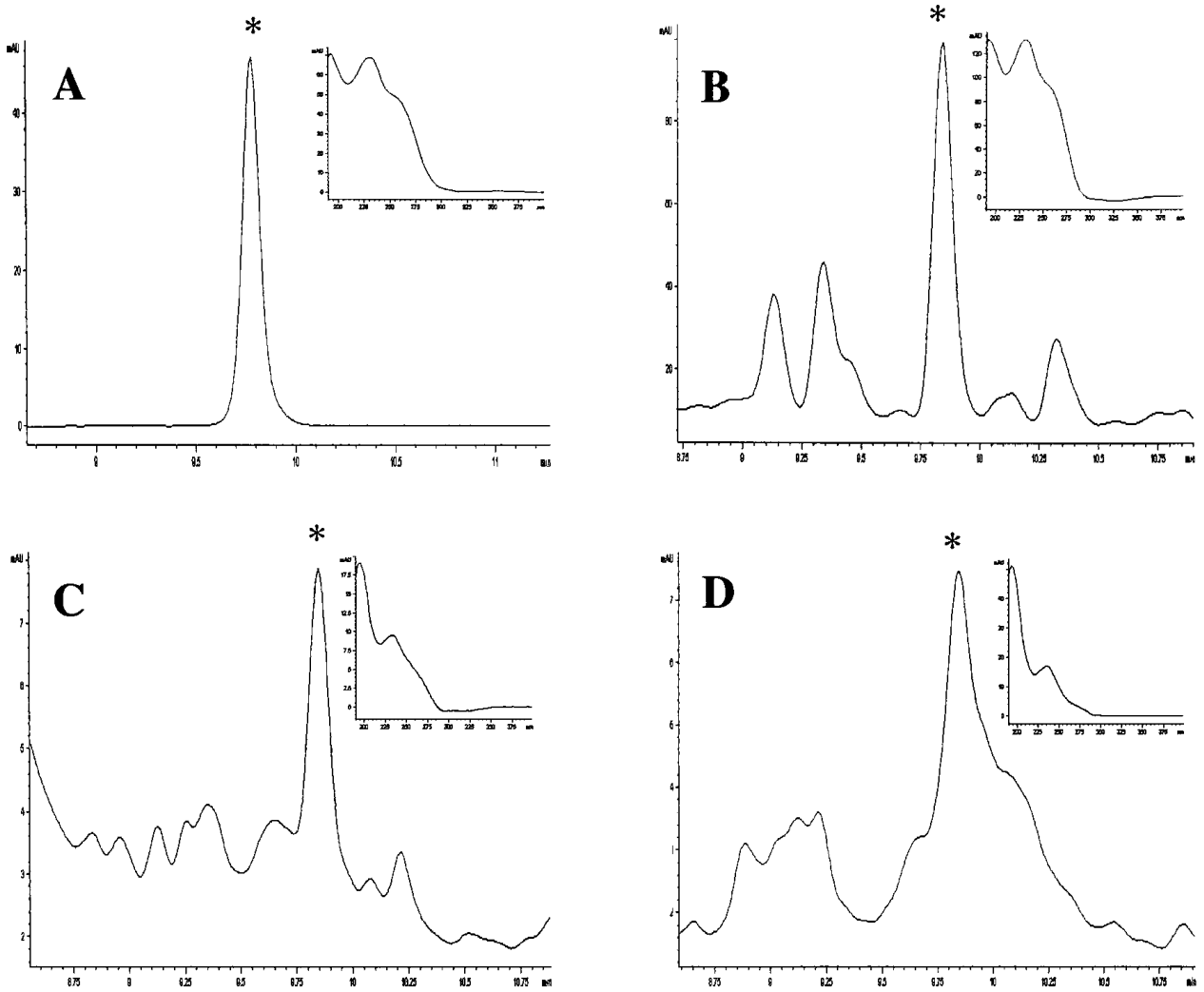


Figure 2.4. HPLC Chromatograms of Filter Extracts from 120-hour Sampling. Retention time is depicted in minutes on the x-axis. Peak sizes in milli-absorbance units (mAU) are shown on the y-axis. For UV spectrum analyses (insets), wavelengths (nm) are shown on the x-axis. Panel A shows a satratoxin H standard, noted with an *, at a concentration of 1 ng/μl with a retention time of 9.777 minutes. The inset shows the UV spectrum of the toxin with a maximum absorbance near 235 nm. Panels B, C, and D are chromatograms of extracts from the 5, 1.2, and 0.4 μm pore size filters, respectively, that were used in a 120-hour sampling. The 5 μm filter extract clearly shows the presence of satratoxin H with a retention time of 9.482 minutes as indicated with an *. UV spectral analysis is also presented (inset). The 1.2 and 0.4 μm filter extract chromatograms show major peaks at 9.845 and 9.848 minutes, respectively, that could be satratoxin H. UV analyses demonstrate that these two peaks are qualitatively similar to purified satratoxin H.

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CHAPTER III

DETECTION OF AIRBORNE *Stachybotrys chartarum* MACROCYCLIC TRICHOTHECENE MYCOTOXINS IN THE INDOOR ENVIRONMENT

Introduction

For the past 25 years, there has been a growing concern about the presence of fungi (with their associated toxic products) and adverse human health effects in indoor environments. In 2002, Dr. Stephen Redd of the Centers for Disease Control made a formal statement before the United States House of Representatives concerning the relationship between indoor molds and human illnesses (35). In this, Dr. Redd stated that "molds can be harmful [and] it is important to maintain buildings, prevent water damage and mold growth, and clean up moldy materials." Additionally, "there remain many unresolved scientific questions" concerning indoor mold contamination, but current research is expanding to include "methods, development, and testing, specifically with regard to state-of-the-art techniques for assessing indoor air quality-related exposures." To date, the majority of indoor air quality investigations have focused on the analysis of mold growth on building materials (1, 18, 31, 37) and measurement of airborne particulate matter including dust, fungal conidia (6, 20) and animal danders (14). These types of investigations cannot accurately assess occupant exposure. Exposure to these factors can influence allergic hypersensitivity responses (2, 10, 27) and symptoms of asthma in certain individuals (12, 28), but most likely does not account for the often reported symptoms such as nausea, dizziness, nose bleeds, physical and mental fatigue, and neurological disorders (26, 36) seen in subjects occupying "sick buildings."

Among the many fungi isolated from contaminated environments, *Stachybotrys chartarum* is one of the most important. *S. chartarum* is a known producer of a number of potent mycotoxins, in particular, the macrocyclic trichothecenes verrucarins B and J, roridin E, satratoxins F, G, and H, and isosatratoxins F, G, and H (21, 25). It has been proposed to be associated with human adverse health effects (9, 13, 15, 23, 24). The members of the macrocyclic trichothecene family of mycotoxins are known to be potent inhibitors of protein synthesis in eukaryotes (16, 34, 44). *S. chartarum* airborne mycotoxins have been studied in various laboratory settings (4, 30, 40, 43) and are known to be detrimental in several animal models (29, 33, 45). However, research that effectively demonstrates the presence of airborne *S. chartarum* trichothecene mycotoxins in native indoor environments is lacking. Studies have mostly focused on detecting these mycotoxins on bulk material (43) or in settled dust (39). For these studies, we hypothesized that airborne trichothecene mycotoxins (notably macrocyclic in nature) exist in indoor environments contaminated with *Stachybotrys chartarum.* A caveat of this was that at any given time, these toxins are present in low concentrations and are therefore difficult to detect. For this reason, an emphasis on the development of simple and rapid collection/detection techniques was employed. Together, these investigations were significant in that the development of a means to collect and detect these mycotoxins would result in a limited occupant exposure.

Materials and Methods

Air Sampling

In each environment, airborne conidia counts (based on five-minute samples) were evaluated prior to and during sampling using either an Allergenco MK-3 non-viable volumetric collector (Allergenco, San Antonio, TX) or a Burkhard Personal Volumetric Air Sampler (Spiral Biotech, Inc., Norwood, MA). The collection characteristics of these two devices have been shown to be similar (32). Conidia were microscopically identified to the genus level. In addition, levels of debris (non-identifiable particles) were qualitatively measured on a scale from very low to very high. Semi-quantification of debris was important because it was composed mostly of highly respirable particles and was viewed as a potential source of mycotoxins (i.e., the particles could serve as carriers of macrocyclic trichothecenes).

For the majority of sampling (seven buildings), air was collected and concentrated from indoor environments using the SpinCon PAS 450-10 bioaerosol sampler (Sceptor Industries, Inc., Kansas City, MO) shown in Figure 3.1. This instrument has been evaluated in the outdoor environment and it has been determined to be a highly effective air sampling device (3, 5). To our knowledge, the SpinCon has never been assessed in indoor environments. The machine operates by continuously pulling air through a glass collector/concentrator at a flow rate of 450 LPM. Entrained solids impact on a sampling liquid (phosphate buffered saline, PBS) and are captured with minimal loss of target particles. Gaseous materials simultaneously dissolve in the fluid droplets. The sampling liquid remains in the collector/concentrator and is continuously concentrated to a final volume of 10 ml. Evaporated sampling fluid is replaced by water in a standardized

manner. After the chosen sampling interval, this fluid was drawn off as a discrete liquid sample that was analyzed in the laboratory. Prior to building sampling, *in vitro* trials were performed whereby air was pulled over cellulose-containing ceiling tiles with confluent *Stachybotrys chartarum* growth directly into the SpinCon collection chamber. This was done to determine if the sampler was capable of collecting airborne macrocyclic trichothecenes originating from *Stachybotrys* growth. These trials were run for 10 and 30 minutes. For comparison purposes, collection was also performed using an equal number of sterile ceiling tiles in the same manner.

Out of the eight fungal-contaminated environments chosen for testing, one was randomly selected to determine if airborne *S. chartarum* trichothecenes existed on particulates smaller than conidia. Sampling was performed using an Andersen GPS-1 Polyurethane Foam (PUF) High Volume Air Sampler (Thermo Electron Corporation, Cheswick, PA). The apparatus was modified to separate and collect particulates smaller than *S. chartarum* conidia through the incorporation of two sterile glass fiber filters of decreasing pore sizes placed in series (Figure 3.2). We have been able to show, using a similar filtration setup in a controlled setting, that conidia can be separated from minute particles that carry trichothecene mycotoxins (4). Large particles were captured using a 90 mm GF/D glass fiber filter (Whatman, Clifton, NJ) with a pore size of 2.7 μm in the upper chamber of the sampling module. Air leaks were prevented by using custom made rubber gaskets (85 and 110 mm inner and outer diameters, respectively). To collect remaining particulate matter, a 90 mm Whatman EPM-2000 glass microfiber filter was fit immediately before the lower chamber of the sampling module. EPM filter material was selected by the United States Environmental Protection Agency as the standard for use in

high volume air sampling. According to the manufacturer, this material is 99.99% efficient for 0.3 μm-sized dioctyl phthalate particles (standard particles for testing filter efficiency) at a 5 cm/sec flowrate. A metal screen was placed immediately after the filter to disperse the air pressure and prevent puncture and/or tearing. Sampling was performed for various times at a flow rate of 150 LPM. The flow rate was based on a calibration curve that was determined with the filters in place using a manometer attached to the sampling module. Following sampling, the filters were removed from the apparatus and transported to the laboratory for analysis.

Indoor Environments and Sampling Conditions

For fungal-contaminated areas, the use of personal protective equipment (PPE) was observed. This included impervious full-body suits (Sunrise Industries, Inc., Guntersville, AL), full face piece respirators (3M, St. Paul, MN) equipped with organic vapor/acid gas cartridge/P100 filter cartridges (3M 60923) or a combination of the two. A total of eight fungal-contaminated indoor environments throughout the state of Texas were chosen for sampling. These buildings had varying degrees of visible fungal growth and water damage. Occupant complaints ranged from minor (musty odors) to severe (loss of mental awareness, dizziness, persistent headaches, etc.). Fungi growing on visible surfaces were sampled and identified using the adhesive tape technique (41). Rooms chosen for testing were based on the degree of visible fungal contamination and/or occupant complaints. Basic descriptions of each test building are outlined in Table 3.1. Further descriptions are detailed in Appendix C.

For buildings sampled using the SpinCon PAS 450-10, air collection was performed at an elevation of 3-4 feet above the ground. Air was sampled in each room under static and/or disturbed conditions for 10, 20, 30, and/or 120 minutes. For those areas sampled with disturbance, 20-inch box fans (Lasko Products, Inc., West Chester, PA) set on "high" were placed in a manner that would circulate the air in the room (one in each corner of the room). The fans were allowed to run for five minutes prior to collection for initial generation of particulate matter. To assess airborne conidia types and concentrations, a five-minute volumetric spore trap was obtained from each room. For static conditions, these samples were taken just prior to sampling. For disturbed conditions, the spore traps were collected five minutes prior to the end of the collection. Four control buildings (i.e., no visible fungal contamination or history of water incursion events) were sampled under similar conditions. In addition, four outside air samples (representing two separate climate regions of Texas) were taken using the SpinCon for 30, 60, 90, and 120 minutes. The final test building and one control building were sampled under static conditions for 24, 48, and/or 72 hours using the Andersen PUF sampler that collected air at a height of approximately five feet. Airborne conidia counts and types were assessed just prior to and immediately following sampling.

Sample Preparation

Following collection, SpinCon samples were filtered using Fisherbrand 13 mm diameter nylon syringe filters with a 0.45 μm pore size (Fisher Scientific, Hampton, NH). The filtered fluid was transferred to 15 ml polypropylene conical centrifuge tubes, frozen at -80°C, and lyophilized using a VirTis Freezmobile (SP Industries, Inc., Gardiner, NY).

The dried samples were individually resuspended in 1 ml of pyrogen-free water for testing.

Filters obtained from the Andersen PUF Sampler were removed and transferred individually to 50 ml polypropylene centrifuge tubes. The filters were suspended in 40 ml of PBS, vortexed vigorously for 60 seconds, and then removed from the tubes. The PBS extracts were then filtered (as described above) into new 50 ml tubes. These were frozen at -80°C, lyophilized, and resuspended in 1 ml of pyrogen-free water for testing.

Macrocyclic Trichothecene Analysis

Samples were analyzed for macrocyclic trichothecenes using the QuantiTox Kit for trichothecenes (EnviroLogix, Portland, ME) as outlined by the manufacturer. This competitive ELISA kit incorporates macrocyclic trichothecene-specific antibodies immobilized on polystyrene microtiter wells (7). All reagents and antibody-coated wells were allowed to equilibrate to room temperature before use. For testing, samples or control mixtures were added to wells in triplicate. Following the incubation, wells were read at 450 nm using an EL-312 microtiter plate reader (Bio-Tek Instruments, Winooski, VT).

ELISA Interpretation

To obtain relative trichothecene concentrations, an ELISA-based macrocyclic trichothecene standard curve was developed (Figure 2.3). Briefly, a mixture of four macrocyclic trichothecenes (satratoxins G and H, verrucarin A, and roridin A) in equal amounts was diluted to 12 test concentrations (500 to 0.1 ng/ml) and tested via the

ELISA as already described. Average ELISA absorbances at 450 nanometers were plotted against toxin concentrations to generate a standard curve. Using this curve, an approximate trichothecene amount was determined for each sample (in ng/ml). Taking into account the collection rate of the air samplers and assuming 100% air sampling efficiency, a semi-quantitative estimate of the amount of airborne trichothecenes for each tested area was then determined (in pg/m^3).

Cross-reactivities to fungal extracts and allergens were expressed as percent inhibitions. These were derived from raw data and were based on test samples versus controls. The percent inhibition represents the degree of inhibition the test sample had on the capability of the satratoxin G-HRP conjugate to bind to the immobilized antibody. They were calculated as done by Schick et al., (38) using the following equation:

$$\% \text{ Inhibition} = 100 \times 1 \quad [(O.D._{450} \text{ sample} - \text{background})/(O.D._{450} \text{ control-background})]$$

Fungal Conidia and Indoor Allergen Cross-reactivity

The composition of indoor air is complex in that numerous types of particulates (fungal conidia, bacteria, animal dander, etc.) are present at any given time. Because of this, we tested our detection methods (ELISA) against some of the most common indoor air constituents. The following fourteen strains of fungi were tested: *Stachybotrys chartarum* (ATCC 201212, IBT 9633, IBT 9757, IBT 9293, IBT 9290), *Alternaria alternata*, *Aspergillus niger* (ATCC 10575), *Chaetomium globosum* (ATCC 16021), *Cladosporium cladosporioides*, *Fusarium sporotrichioides* (ATCC 24630), *Memnoniella echinata* (ATCC 11973), *Penicillium chrysogenum*, *Trichoderma viride* and one species

of *Rhizopus*. Out of the five *S. chartarum* strains tested, only strain 201212 produced macrocyclic trichothecenes. Fungi not purchased from a supplier were collected from outside samples, purified, and identified in our laboratory according to Sutton et al., (42) and de Hoog et al., (11). All fungi were maintained on potato dextrose agar (BD Diagnostic Systems, Sparks, MD) on 90 mm plastic Petri dishes in a controlled 25°C incubator (Fisher Scientific Isotemp Incubator, Model 304). For testing purposes, conidia were collected from plates that had reached confluence using sterile cotton swabs. The cotton tips of the swabs were placed in 1 ml of sterile PBS and vortexed vigorously to remove conidia. The conidia were then counted using a hemacytometer and diluted to 1 x 10^6, 10^5, and 10^4 conidia/ml of PBS constituting the working solutions for ELISA testing.

The three common indoor allergens Can f 1, Der p 1, and Fel d 1 (Indoor Biotechnologies, Charlottesville, VA) were also tested. For ELISA testing purposes, the allergens were individually diluted in PBS to 50 ng/ml, 5 ng/ml, and 500 pg/ml.

Statistical Analysis

Statistical analyses were performed on individual collected samples using Sigma Stat 2.0 software (Chicago, IL). Samples were grouped into control and fungal-contaminated environments by sampling times and conditions. Airborne macrocyclic trichothecene equivalents were compared and are reported here. Mold-contaminated areas (test buildings) where *Stachybotrys chartarum* was not clearly identified using our survey methods were excluded from statistical analyses as they were not applicable for trichothecene determination. These represented a small population of the total rooms

sampled (five rooms). Thirty-minute samples (N=15) and two hour samples (N=27 under static conditions, 9 under disturbed conditions) were compared to two hour static controls (N=69) using a Kruskal-Wallis One Way Analysis of Variance (ANOVA) on Ranks. Ten-minute test samples taken under disturbed conditions (N=24) were compared to similarly sampled controls (N=9) using a Mann-Whitney Rank Sum Test. Statistical significance for these analyses was reported as having a P-value of <0.05.

Data obtained from samples using the Andersen PUF sampler were normalized and compared using a one-way ANOVA followed by Tukey post-hoc analysis (N=3 for each filter type and collection period). Test filters were compared to corresponding filter types that were used for 24-hour sampling in a control environment. Statistical significance was defined as having a P-value of less than 0.05.

All ELISA cross-reactivities (as percent inhibitions) for fungal extracts were compared to PBS alone using a Kruskal-Wallis one way ANOVA on ranks (P = <0.05). Data from each organism were then normalized and grouped based on conidia concentrations. These were compared to the macrocyclic trichothecene-producing strain of *S. chartarum* using a one way ANOVA (P<0.05). Allergens were compared to PBS alone using a one-way ANOVA (P<0.05).

Results

Macrocyclic Trichothecene Analysis

Table 3.2 shows data from three of the fungal-contaminated environments and two of the control buildings. Detailed analyses of all sampled buildings (images, airborne conidia types and counts, and ELISA results) are shown in Appendices C and D.

A general trend was seen showing that as airborne *Stachybotrys* conidia and/or debris counts increase, so do airborne trichothecene mycotoxins concentrations. These areas were representative of all of the samples taken in regards to sampling times and conditions, range of water damage and fungal contamination, and effects of remediation on air quality. For example, samples taken in the first room of Test Building 1 demonstrated that with short controlled disturbance periods, airborne trichothecene concentrations increase. Alternatively, longer disturbance periods (30 minutes and two hours) resulted in lower concentrations of collected mycotoxins. Airborne macrocyclic trichothecene concentrations also greatly increased during aggressive sampling as seen in Test Building 2. Data from Test Building 3 demonstrate the specificity of our detection methods. Although this area was heavily contaminated with fungi (*Memnoniella*, *Alternaria*, and others) no *Stachybotrys* was identified in the sampled areas resulting in airborne trichothecene equivalents similar to controls. This was also the case for room 3 of Test Building 1 where *Chaetomium* was the primary contaminant. These areas were excluded from statistical analyses. No control building was completely negative and trichothecene equivalents increased during short disturbance periods.

Airborne trichothecene concentrations from all of the sampled buildings are summarized in Figure 3.3. Collective percent inhibitions are shown in Appendix D (Figure D.1). Overall, detectable levels of airborne macrocyclic trichothecene mycotoxins were significantly ($P<0.05$) higher in *S. chartarum*-contaminated buildings versus control buildings. The median values were 3.9, 9.0, 7.5, and 61.5 pg/m^3 of air for two-hour static controls and tests, two-hour disturbed tests, and 30-minute disturbed tests,

respectively. The median values were 44.9 and 248.5 pg/m^3 of air for the 10-minute controls and tests, respectively.

Outside air, regardless of the sampling time, was always negative (Appendix D, Table D.2). We demonstrated significant collection of macrocyclic trichothecene mycotoxins from *S. chartarum* using an *in vitro* setup in as little as 10 minutes. Sterile ceiling tile alone in this setup also showed ELISA reactivity for trichothecene mycotoxins, but at significantly lower levels than when *Stachybotrys* was present (Appendix D, Table D.2).

Table 3.3 shows results from the two buildings that were sampled using the Andersen PUF high volume sampler. Data are presented similarly to Table 3.2, but with an additional column noting the glass microfiber filter used. For each sampling time in the test building, the 2.7 μm pore size filter (GF/D) in the setup demonstrated higher ELISA reactivity than the second (EPM) filter. Values obtained in the test environment regardless of filter type were significantly ($P<0.05$) higher than those obtained in the control environment. In fact, results seen on the GF/D filter used in the one control room tested with the PUF sampler were similar to negative controls tested with the SpinCon. The EPM filter demonstrated no significant ELISA reactivity in this control environment.

Fungal Conidia and Indoor Allergen Cross-reactivity

ELISA cross-reactivity data to 14 frequently isolated indoor fungi and three common indoor allergens are shown in Table 3.4. Data are expressed in percent inhibitions as already described. ELISA cross-reactivity testing with the 13 strains that do not produce macrocyclic trichothecenes ranged from negligible to minimal. Only *S.*

chartarum strain 201212 was significantly ($P<0.05$) different than the extracting solvent (PBS) alone. This was seen for all three tested conidia concentrations. Low percent inhibitions were seen with the four IBT strains of *S. chartarum* and the *Chaetomium globosum* strain at the highest concentrations tested ($1x10^6$ conidia/ml). These results were significantly ($P<0.05$) lower than those obtained with the macrocyclic trichothecene producing strain of *S. chartarum*. Can f 1, Der p 1, and Fel d 1 did not demonstrate cross-reactive binding.

Discussion

In this study, through the use of two types of high volume air samplers in conjunction with sensitive and specific ELISA technology, we were successful in demonstrating airborne macrocyclic trichothecene mycotoxins in eight *Stachybotrys chartarum*-contaminated indoor environments. As Table 3.1 shows, there was a considerable range in water damage and mold contamination in the eight test buildings. Similarly, self-reported occupant health complaints varied from building to building. This was expected because no widely accepted methods for measuring indoor mold related health issues currently exist. Although health complaints were not quantified, we were able to show as mold (specifically *S. chartarum*) presence increases, the number and degree of occupant complaints rises. The airborne concentrations of macrocyclic trichothecenes in the *Stachybotrys*-contaminated indoor environments tested were between 10 and 1300 pg/m^3 of air. Animal models have demonstrated that lethal doses for trichothecenes are relatively low (8) and toxicity is greater via the respiratory route

(44). These studies focused primarily on non-macrocyclic trichothecenes which are known to be less toxic than the macrocyclic trichothecenes produced by *S. chartarum* (44). It is therefore feasible that the low levels found in the buildings we tested could adversely affect occupant health in a chronic manner. To date, however, there have been no described methods or standards relating human health and airborne macrocyclic trichothecene exposure in indoor mold-contaminated environments.

Seven of the eight *Stachybotrys*-contaminated environments were tested using the SpinCon PAS 450-10 high volume wet concentration bioaerosol sampler. Our data demonstrated an increase in airborne mycotoxin concentrations in relation to increased conidia and debris counts. This was an expected result since the two primary mycotoxins produced by *S. chartarum* (satratoxins G and H) are known to be associated primarily with conidia (19) and consequently, fungal fragments (4, 17). In a similar manner, aggressive sampling (invasive inspection) results in extensive release of airborne particulate matter including conidia and mycotoxins. This poses a potential health risk to remediators and emphasizes the need for PPE while working in mold-contaminated areas. The mechanisms used to disturb the air for these experiments were a means to shorten the overall sampling times. Such an intense disturbance and consequent release of particulates inclusive of *S. chartarum* conidia would not be expected to occur in native environments. However, subtler disturbance mechanisms (human and mechanical vibrations, ceiling fans, air conditioning units, etc.) are hypothesized to cause the persistent release of such particulate matter over a longer period of time. This supports the idea that adverse human health effects in mold-contaminated buildings are a result of chronic more often than acute exposure. Longer disturbance under intense conditions

resulted in lower trichothecene concentrations. This was most likely due to the physical exiting of airborne trichothecenes from the sampled room before the SpinCon could collect them. Taken as a whole, we believe we have developed a new means of assessing indoor air quality. Based on our data, this would involve an initial two-hour or longer static sample to evaluate airborne contamination in the native setting. This would be followed by a much shorter sampling period (ideally 10 minutes) under air disturbance conditions to assess the potential for trichothecenes to become airborne in that environment.

Our testing methods were specific for buildings contaminated with macrocyclic trichothecene-producing strains of *S. chartarum*. The specific nature of our methods was seen in both controlled and natural settings. For controlled analyses, ELISA cross-reactivity was tested with 13 strains of fungi (frequently isolated from indoor environments) that do not produce macrocyclic trichothecenes. Cross-reactivities ranged from negligible to minimal. The four IBT strains of *S. chartarum* and the one *Chaetomium globosum* strain demonstrated uncharacteristically high ELISA reactivities at $1x10^6$ conidia/ml. These were higher than the average for all of the fungi tested, but were still not significant in comparison to the macrocyclic trichothecene producing strain of *S. chartarum*. For the *Stachybotrys* strains, this could be due to a basal level of trichothecene production not detectable by previously described analytical methods (21, 22, 25). Compounds produced by *C. globosum* have been poorly studied. It is therefore possible that this organism produces trichothecenes or similarly structured compounds. Because the antibody used for detection is against a small chemical compound, non-specific binding to compounds with similar structures (haptens) is possible. This is of

particular importance when considering the complex composition of indoor air. This may also be a reason why we detected low levels of trichothecenes in negative control environments.

As mentioned, the specificity of our testing was also shown in natural settings. Certain areas (room 3 in Test Building 1 and all of Test Building 3) were heavily contaminated with fungi; however, results obtained from sampling in this building were similar to negative controls. Even when high concentrations of other fungi were present (such as *Chaetomium* and/or *Memnoniella*), positive results were only seen in environments contaminated with macrocyclic trichothecene-producing *Stachybotrys* strains during the time of sampling.

The eighth building was unique in that we tested the hypothesis that airborne trichothecene mycotoxins were present on particulates smaller than fungal conidia. This is important because in the indoor environment, fragments and other highly respirable particles greatly outnumber intact fungal conidia (17). Current accepted techniques for assessing indoor air quality generally do not take these potential health hazards into consideration. Instead, most investigations rely on bulk sampling and viable/non-viable airborne conidia assessments. Previously, in a controlled filtration setup (Figure 2.1), we were able to demonstrate *S. chartarum* trichothecene mycotoxins on particles smaller than conidia (4). In the current study, we were able to show this same phenomenon after 24, 48, and 72 hours of high volume air sampling in a native mold-contaminated building. These findings indicate the need to collect and analyze this class of particulates when conducting indoor air quality investigations.

Our study shows that macrocyclic trichothecene mycotoxins from *Stachybotrys chartarum* can become airborne in indoor environments contaminated with this organism. Our data suggest the need to test for these potential occupant health risks during indoor air quality investigations. Although we were able to semi-quantitate airborne concentrations, it is still not known what levels of these mycotoxins pose a definitive human health risk. Additionally, "normal" background levels (if they do exist) have not been characterized. Future research should focus on the relationship between respiratory exposure to airborne trichothecenes in fungal-contaminated buildings and human health issues resulting from such exposures.

Table 3.1. Test building descriptions.

Building	Age[a]	Occupant Complaints[b]	Visible Water Damage[c]	Total Visible Fungal Contamination[c]	*Stachybotrys* Identification[d]	
					Surface	Airborne
1	10	Musty odor, eye/throat irritation, nosebleeds, rash/itchy skin, headaches, fatigue, loss of mental awareness, dizziness, nausea	200-300	10-50	Yes	Yes
2	10	Musty odor	>500	>500	Yes	Yes
3	10	None	1-10	<1	Yes	Yes
4	>20	None	1-10	<1	Yes	Yes
5	>20	Musty odor, eye/throat irritation, nosebleeds, headaches	50-100	50-100	No	Yes
6	>20	Musty odor, eye/throat irritations, sneezing, sinus congestion	100-200	10-50	Yes	No
7	>20	Musty odor	>500	200-300	No	No
8	>20	Musty odor, eye/throat irritations, nosebleeds, headaches	100-200	100-200	Yes	No

[a]Approximate age (in years) during the time of sampling.

[b]Self-reported complaints during the time of occupation. Contamination increased in Buildings 1, 2, 7, and 8 after occupants permanently vacated the premises.

[c]Estimation in square feet. Further fungal contamination was noted for each environment following invasive inpection/remediation.

[d]Surface growth was assessed using the adhesive tape technique described in the text. Airborne *Stachybotrys* conidia concentrations were determined using impaction spore traps that are described in the text.

Table 3.2. Individual data points from representative sampled buildings.

Building	Room	Dimensions[a]	Sampling Time and Conditions[b]	Spore Trap Counts[c]		Average Macrocyclic Trichothecene Equivalents/m^3 of Sampled Air[d]
				Conidia/m^3	Debris	
Test 1	1	20 x 25 x 10	120 min Static	1226	Heavy	36.7 ± 4.7
			120 min Disturbed	5032	Medium	7.7 ± 0.2
			30 min Disturbed	0	Very Light	159.3 ± 32.1
			10 min Disturbed	2710	Medium	1172.0 ± 145.2
	2	20 x 25 x 8	120 min Static	0	Medium	5.2 ± 0.3
			120 min Disturbed		Medium	3.7 ± 0.4
			30 min Disturbed		Light	18.8 ± 1.3
			10 min Disturbed		Medium	42.9 ± 5.1
	3	25 x 30 x 8	120 min Static	0	Very Light	4.6 ± 0.4
			30 min Disturbed	0	Very Light	18.3 ± 0.4
			10 min Disturbed	452	Medium	85.9 ± 9.4
Test 2	1	6 x 8 x 8	120 min Static	8839	Medium	4.8 ± 0.3
			10 min Agg 1	16968	Very Heavy	Above Scale
			10 min Agg 1 (1:10)	-	-	355.0 ± 65.6
			10 min Agg 2	14355	Very Heavy	Above Scale
			10 min Agg 2 (1:10)		-	756.7 ± 99.5
			10 min Agg 3	THTC	Very Heavy	Above Scale
			10 min Agg 3 (1:10)	-		1368.1 ± 144.9
Test 3	1	8 x 90 x 8	120 min Static	0	Light	3.7 ± 0.3
			20 min Disturbed		Medium	20.7 ± 1.9
	2	15 x 15 x 8	120 min Static		Light	3.1 ± 0.2
			20 min Disturbed		Heavy	21.7 ± 1.4
	3	8 x 90 x 8	120 min Static		Light	3.2 ± 0.4
			20 min Disturbed		Light	19.7 ± 0.5
	4	15 x 15 x 8	120 min Static		Light	4.3 ± 0.2
			20 min Disturbed		Medium	21.7 ± 1.0

Table 3.2. Continued.

Building	Room	Dimensions[a]	Sampling Time and Conditions[b]	Spore Trap Counts[c]		Average Macrocyclic Trichothecene Equivalents/m^3 of Sampled Air[d]
				Conidia/m^3	Debris	
Control 1	1	15 x 20 x 10	120 min Static	0	Light	5.3 ± 0.5
	2	30 x 20 x 10			Light	3.3 ± 0.9
	3	20 x 20 x 10			Very Light	5.0 ± 0.3
	4	20 x 20 x 10			Light	3.4 ± 0.7
	5	25 x 20 x 10			Very Light	3.9 ± 0.2
Control 2	1	15 x 12 x 8	120 min Static	0	Medium	4.6 ± 0.1
			10 min Disturbed		Medium	36.1 ± 6.2
	2	15 x 12 x 8	120 min Static		Medium	5.4 ± 2.4
			10 min Disturbed		Light	75 8 ± 32.8
	3	20 x 20 x 8	120 min Static		Light	4.0 ± 0.3
			10 min Disturbed		Medium	49.9 ± 5.2

[a]Length x width x height (in feet).

[b]Rooms were sampled under static and/or disturbed conditions for 10, 20, 30, or 120 minutes as noted. Air disturbance was accomplished using 20-inch box fans on a "high" setting. Disturbance was allowed for 5 minutes prior to starting the SpinCon collection. Test building 2 is a representative of the environments that were sampled during aggressive sampling (Agg).

[c]5-minute Allergenco spore traps were taken to assess airborne fungal conidia types and concentrations as well as to qualitate the amount of debris present. For static conditions, samples were taken just prior to SpinCon collection. For disturbed conditions, Allergencos were started 10 minutes prior to the end of the sampling period. Only *Stachybotrys* counts are presented here. Debris was defined as non-identifiable particles and were qualitated based on the approximate percentage of the viewed field covered by such particles: Very Light (<20%), Light (21-40%), Medium (41-60%), Heavy (61-80%), and Very Heavy (>80%, unable to read). THTC; too heavy to count

[d]In picograms. Means ± standard deviations of triplicate wells are shown. Estimated values are based on the average trichothecene equivalents for the entire collected sample, collection time, and flow rate of the SpinCon. For example, a total of 54 m^3 of air was collected for each 120-minute sample. Given a final working volume of 1 milliliter, airborne trichothecene concentrations were then extrapolated from an ELISA-based macrocyclic trichothecene standard curve (Figure 2.3). Values represent triplicate wells.

Table 3.3. Air sampling analyses from Andersen-sampled test and control indoor environments.

Building	Dimensions.[a]	Filter Type[b]	Sampling Time[c]	Spore Trap Counts[d]		Average Macrocyclic Trichothecene Equivalents/m^3 of Sampled Air[e]
				Conidia/m^3	Debris	
Test Building 8	3 x 6 x 8	GF/D	24	0	Light	62.6 ± 12.3*
		EPM				6.9 ± 2.1*
		GF/D	48			14.3 ± 2.0*
		EPM				1.8 ± 0.2
		GF/D	72			25.1 ± 1.4*
		EPM				4 1 ± 0.6*
Control Building 1	20 x 10 x 10	GF/D	24	0	Very Light	1.1 ± 0.1
		EPM				1.9 ± 0.09

[a]Length x width x height (in feet).

[b]Glass microfiber filters. GF/D filters have a 2.7 mm pore size. EPM filters are 99.99% efficient for 0.3 mm particles and are described in the text. These filters were situated as depicted in Figure 3.2.

[c]In hours

[d]5-minute Allergenco spore traps were taken to assess airborne fungal conidia types and concentrations as well as to qualitate the amount of debris present. For static conditions, samples were taken just prior to SpinCon collection. For disturbed conditions, Allergencos were started 10 minutes prior to the end of the sampling period. Only *Stachybotrys* counts are presented here. Debris was defined as non-identifiable particles and were qualitated based on the approximate percentage of the viewed field covered by such particles: Very Light (<20%), Light (21-40%), Medium (41-60%), Heavy (61-80%), and Very Heavy (>80%, unable to read). THTC; too heavy to count

[e]In picograms. Means ± standard deviations of triplicate wells are shown. Estimated values are based on the average trichothecene equivalents for the entire collected sample, collection time, and flow rate of the Andersen. For example, a total of 216 m^3 of air was collected for a 24-hour sample. Given a final working volume of 1 milliliter, airborne trichothecene concentrations were then extrapolated from an ELISA-based macrocyclic trichothecene standard curve (Figure 2.3). Values determined to be significantly different ($P<0.05$) from corresponding filter types in the control environment are noted with an *. Values represent triplicate wells.

Table 3.4. Competitive ELISA inhibition of commonly isolated indoor fungi and allergens.

Sample[a]	Concentration[b]	Average % Inhibition[c]
Stachybotrys chartarum (ATCC 201212)	1 x 10^6 conidia/ml	95.69 ± 0.58
	1 x 10^5 conidia/ml	95.63 ± 0.03
	1 x 10^4 conidia/ml	90.76 ± 0.52
Stachybotrys chartarum (IBT 9633)	1 x 10^6 conidia/ml	24.19 ± 9.47
	1 x 10^5 conidia/ml	6.69 ± 3.26
	1 x 10^4 conidia/ml	2.41 ± 4.17
Stachybotrys chartarum (IBT 9757)	1 x 10^6 conidia/ml	8.44 ± 3.58
	1 x 10^5 conidia/ml	11.61 ± 10.05
	1 x 10^4 conidia/ml	10.62 ± 4.41
Stachybotrys chartarum (IBT 9293)	1 x 10^6 conidia/ml	22.23 ± 4.24
	1 x 10^5 conidia/ml	15.39 ± 1.42
	1 x 10^4 conidia/ml	11.97 ± 4.99
Stachybotrys chartarum (IBT 9290)	1 x 10^6 conidia/ml	29.75 ± 1.26
	1 x 10^5 conidia/ml	10.64 ± 9.29
	1 x 10^4 conidia/ml	23.99 ± 22.79
Alternaria alternata	1 x 10^6 conidia/ml	15.00 ± 14.62
	1 x 10^5 conidia/ml	15.12 ± 5.60
	1 x 10^4 conidia/ml	14.88 ± 10.69
Aspgerillus niger (ATCC 10575)	1 x 10^6 conidia/ml	22.26 ± 18.44
	1 x 10^5 conidia/ml	0.0
	1 x 10^4 conidia/ml	4.33 ± 3.84
Chaetomium globosum (ATCC 16021)	1 x 10^6 conidia/ml	29.84 ± 3.87
	1 x 10^5 conidia/ml	6.13 ± 7.10
	1 x 10^4 conidia/ml	9.60 ± 9.09
Cladosporium cladosporioides	1 x 10^6 conidia/ml	0.00 ± 0.00
	1 x 10^5 conidia/ml	7.07 ± 5.56
	1 x 10^4 conidia/ml	7.10 ± 8.31

Table 3.4. Continued.

Sample[a]	Concentration[b]	Average % Inhibition[c]
Fusarium sporotrichioides (ATCC 24630)	1 x 10^6 conidia/ml	0.0
	1 x 10^5 conidia/ml	0.0
	1 x 10^4 conidia/ml	0.0
Memnoniella echinata (ATCC 11973)	1 x 10^6 conidia/ml	3.65 ± 6.32
	1 x 10^5 conidia/ml	2.31 ± 3.23
	1 x 10^4 conidia/ml	4.75 ± 7.49
Penicillium chrysogenum	1 x 10^6 conidia/ml	0.0
	1 x 10^5 conidia/ml	1.82 ± 3.15
	1 x 10^4 conidia/ml	0.0
Rhizopus sp.	1 x 10^6 conidia/ml	4.23 ± 5.71
	1 x 10^5 conidia/ml	6.20 ± 5.07
	1 x 10^4 conidia/ml	7.07 ± 8.34
Trichoderma viride	1 x 10^6 conidia/ml	11.70 ± 5.87
	1 x 10^5 conidia/ml	0.00 ± 0.00
	1 x 10^4 conidia/ml	1.56 ± 2.71
Can f 1	50 ng/ml	0.0
	5 ng/ml	0.0
	500 pg/ml	0.0
Der p 1	50 ng/ml	0.0
	5 ng/ml	0.0
	500 pg/ml	0.0
Fel d 1	50 ng/ml	0.0
	5 ng/ml	0.0
	500 pg/ml	0.0

[a]Cultures were grown in 90 mm petri dishes on potato dextrose agar at 25°C. Catalog numbers for purchased fungi are noted. All other fungal samples were obtained from outside sources and identified microscopically in our laboratory.

[b]Fungal conidia were collected from the surfaces of 14-day-old cultures using sterile cotton swabs. The cotton tips of the swabs were placed in 1 ml of sterile pyrogen-free water and vortexed to remove conidia. Conidia were counted using a hemacytometer and diluted to the above concentrations in sterile water. Purified allergens (in 50% glycerol) were diluted in phosphate buffered saline to the noted concentrations.

[c]Means ± standard deviations are shown. Negative inhibitions were converted to 0.0% inhibition.

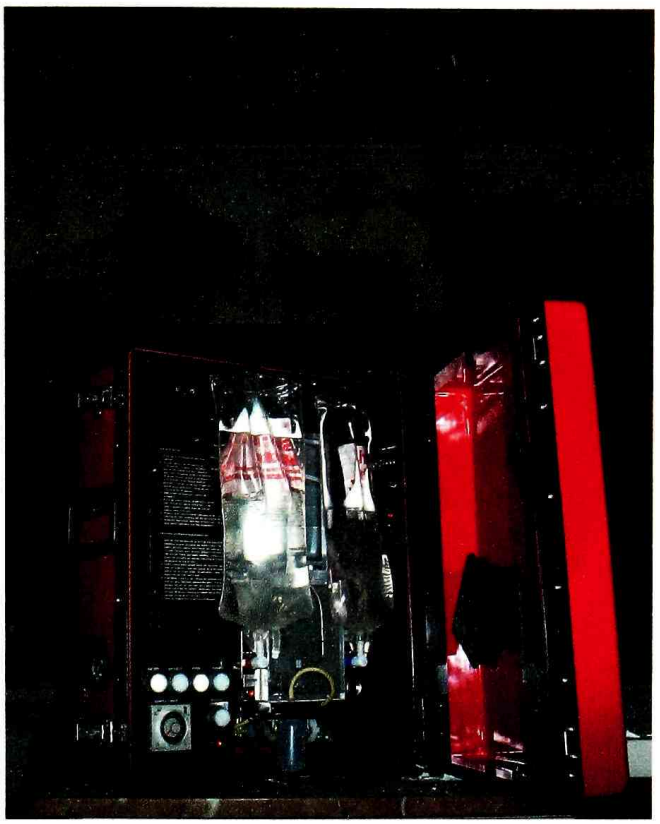

Figure 3.1. SpinCon PAS 450-10 Bioaerosol Sampler Setup. The SpinCon bioaerosol sampler was used in Buildings 1-7. The air intake pulls in air at a flowrate of 450 LPM. Particles are collected and concentrated in PBS (left hanging bag) while evaporated fluid is constantly replaced (right hanging bag) with sterile water. Samples were collected in a final volume of 10 ml. This image was taken in a *Stachybotrys chartarum*-contaminated building (Test Building 2). The fungal growth is clearly evident on the walls and ceiling in the background of this picture.

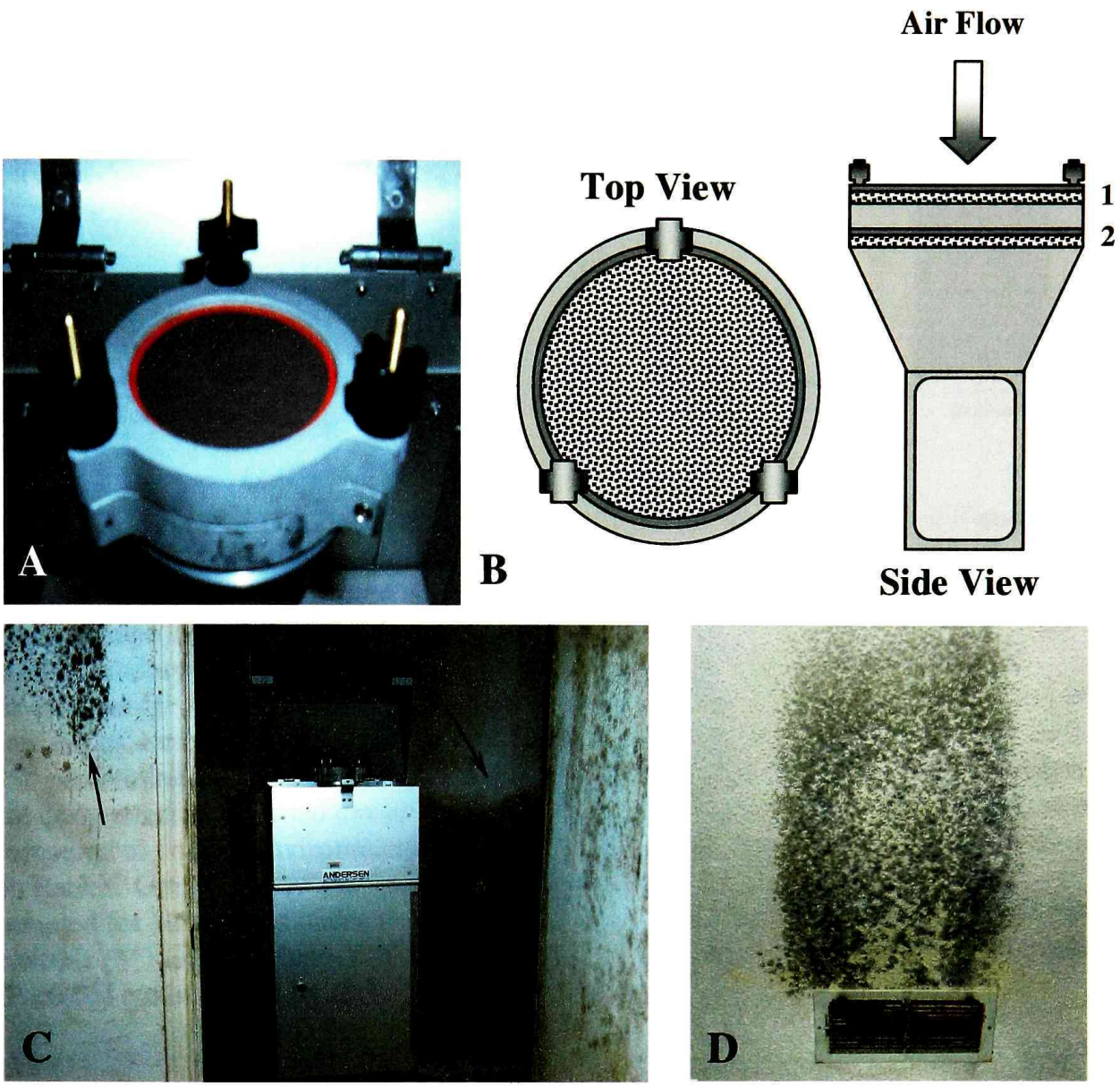

Figure 3.2. Andersen GPS-1 PUF High Volume Air Sampler Setup. Panel A shows the collection module following a 24-hour sampling period. The top filter with a considerable amount of collected particulates is visible here. Panel B is a schematic of the collection module with a top view on the left and a side view on the right. The module was modified to collect and separate particles using glass microfiber filters. Large particles, including most fungal conidia, were collected on 90 mm diameter 2.7 µm pore size GF/D filters (1) while remaining particles able to pass through the first filter were collected on highly efficient EPM filters of the same diameter (2). A heavily mold-contaminated storage closet adjacent to the source of the water damage (shown in panel C) was chosen for the sampling. Water-saturated air and ensuing fungal contamination was a result of major damage to the air conditioning unit. The degree of the damage was evident by growth near the air exit grates throughout the building (panel D).

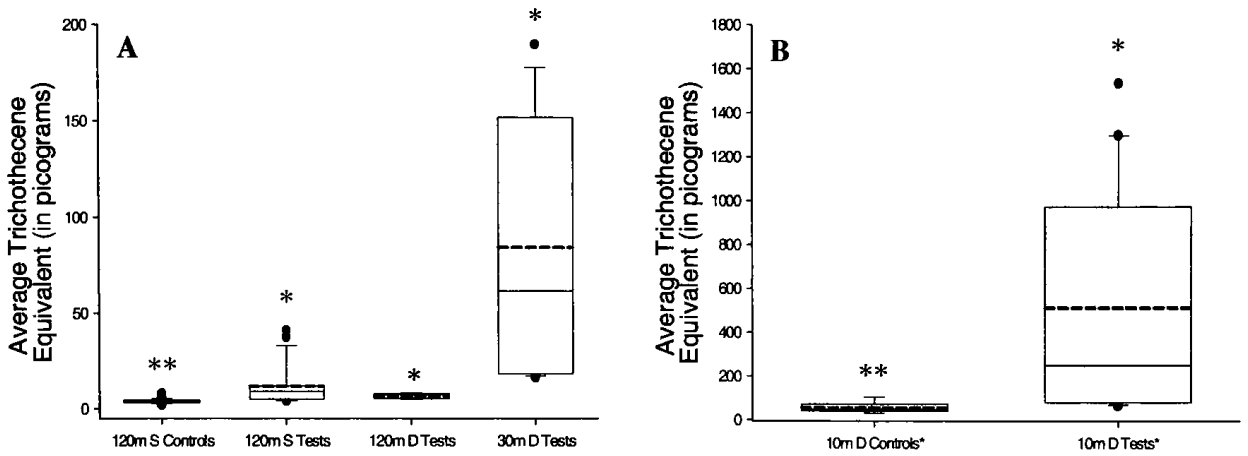

Figure 3.3. Box Plot Data for Average Trichothecene Equivalents per m^3 of Sampled Air in *Stachybotrys*-Contaminated and Control Indoor Environments. Trichothecene equivalents (in picograms) were determined using a macrocyclic trichothecene standard curve. Graph A shows cumulative data obtained from 120 minute (m) control and test samples under static (S) and disturbed (D) conditions and 30-minute disturbed test environments. Medians (solid line) and means (dotted line) are shown. The 10^{th} and 90^{th} percentiles are designated by the bottom and top error bars, respectively. The 25^{th} and 75^{th} percentiles are indicated by the bottom and top of the boxes, respectively. Outliers are designated as the filled circles above and/or below the plot. Test environments were compared to control environments using a Kruskal-Wallis One Way Analysis of Variance on Ranks. Graph B shows cumulative results from control and test environments sampled for 10 minutes under disturbed conditions. Test environments were compared to controls (**) using a Mann-Whitney Rank Sum Test. Statistically significant differences ($P < 0.05$) are indicated by an *.

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CHAPTER IV

DETECTION OF TRICHOTHECENE MYCOTOXINS IN SERA FROM INDIVIDUALS EXPOSED TO *Stachybotrys chartarum* IN INDOOR ENVIRONMENTS

Introduction

The relationship between poor indoor air quality (IAQ) and human health has been the focus of many scientific investigations spanning hundreds of years. Poor IAQ and associated adverse human health effects can be the result of numerous factors that include, but are not limited to, poor ventilation/air circulation (41), the presence of volatile organic compounds of microbial and chemical origin (10, 31), allergens such as animal danders (14), and water damage/mold contamination. Of these, water damage and ensuing mold growth is currently one of the most important public issues facing the IAQ field. Problems associated with fungal contamination in indoor environments have been long recognized. As a result, knowledge concerning effects resulting from exposure to fungi has been vast and well-documented. Fungi have been shown to influence allergic hypersensitivity responses in several animal models (6, 7, 40) and in humans (8, 27). Additionally, symptoms of asthma arising from exposure to fungi have been recognized in certain individuals (12, 17, 28, 33). These types of symptoms are important when considering IAQ, but cannot account for the often reported severe symptoms of nausea, dizziness, nose bleeds, physical and mental fatigue, and neurological disorders (11, 15, 26). These types of symptoms may be the result of airborne mycotoxin inhalation (3). Of the toxin-producing fungi most commonly isolated from indoor environments,

Stachybotrys chartarum is considered to be one of the most important with relevance to human health (24).

S. chartarum is a known producer of a number of potent mycotoxins, in particular, the macrocyclic trichothecenes verrucarins B and J, roridin E, satratoxins F, G, and H, and isosatratoxins F, G, and H (21, 25). In addition, it has been proposed to be associated with human adverse health effects (9, 13, 15, 22, 23). The members of the macrocyclic trichothecene family of mycotoxins are known to be potent inhibitors of protein synthesis in eukaryotes (16, 38, 49). *S. chartarum* airborne mycotoxins have been studied in various laboratory settings (2, 32, 42, 45) and are known to be detrimental in several animal models (29, 37, 50). Their presence in natural settings (*S. chartarum* contaminated buildings) has also been demonstrated (3). Currently, however, very few studies exist that demonstrate a relationship between exposure to airborne *S. chartarum* mycotoxins and adverse human health effects (47, 48). For this study, we hypothesized that trichothecene mycotoxins were present and could be detected in the sera of individuals who had been exposed to *S. chartarum* in contaminated indoor environments. Trichothecene mycotoxins detected in said individuals would be another step in the progression showing an association between the presence of *S. chartarum* in buildings and reported human health complaints.

Materials and Methods

Human Serum Samples

All serum samples were received in frozen 1 ml aliquots and de-identified according to the Texas Tech University Health Sciences Center Institutional Review

Board. Samples were divided into three groups: Group 1 consisted of individuals with documented indoor *Stachybotrys* exposure (N=18). In this group, samples 1-11 were selected from a group of individuals working under the same indoor environmental conditions in a water-damaged office building for a period of three years. During physical examination, common symptoms included blurred vision, memory loss, fatigue, headache, nausea, loss of balance, cognitive deficits, rhinitis, sinusitis, nosebleeds, rashes and allergies were recorded. An environmental engineering firm tested the building by tape transfer and swab samples. Heavy *Stachybotrys* contamination (> 5,000 colony forming units/m^3 of sampled air) was reported. Samples 12-14 were taken from three individuals who lived in a heavily water-damaged building for several years. *Stachybotrys* surface contamination was noted and airborne conidia counts were as high as 5000 conidia per cubic meter of sampled air. Reported health complaints included eye/throat irritation, nose bleeds, headaches, dizziness, nausea, and loss of mental awareness. Samples 15-18 were taken from four individuals who dwelled in a building with heavy water damage and numerous fungal growth sites. An indoor air quality firm verified the presence of *Stachybotrys* in air, tape, and bulk samples. Reported health symptoms included nausea, vomiting, diarrhea, muscle aches, frequent nose bleeds, and even hemorrhage and seizures. Group 2 consisted of individuals with reported indoor mold exposure that was not specified (N=26). Detailed demographics, history and symptoms were provided for each sample. Symptoms ranged from minor (congestion, cough, watery eyes, etc.) to relatively severe (nausea, vomiting, dizziness, weakness, numbness, etc.). Group 3 consisted of individuals with no known mold and/or mycotoxin exposure (N=26) and served as negative controls.

Sample Extraction and Preparation

Before testing, serum samples were extracted in a manner similar to that described by Garbis et al. (18) and Hedman et al. (20). This was done as a simple and rapid means to remove high molecular weight proteins that produced an undesirably high background noise in the ELISA testing [data not shown]. Briefly, serum samples were individually aliquoted (200 µl) into sterile 1.5 ml polystyrene microcentrifuge tubes followed by the immediate addition of 600 µl of high performance liquid chromatography (HPLC)-grade acetonitrile. Samples were allowed to sit at room temperature for 15 minutes after which they were vortexed vigorously for 30 seconds. They were then centrifuged at 14,500 RPM for three minutes to pellet the precipitated proteins. The supernatants were individually transferred into clean 1.5 ml glass vials. Each sample was evaporated to completion under a gentle stream of dry nitrogen and resuspended in 200 µl pre-warmed sterile water. The warming aided in the resuspension of samples. This was the final working solution for the ELISA. The acetonitrile extraction step resulted in negligible, if any, loss of potential trichothecene mycotoxins present. This was based on macrocyclic trichothecene-spiked NHS (CELLect® Human Serum, Fisher Scientific, Hampton, NH) samples that were extracted as already described (see below).

Trichothecene Mycotoxin Analysis

Samples were analyzed for trichothecenes using the QuantiTox Kit for trichothecenes (EnviroLogix, Portland, ME) as outlined by the manufacturer. This competitive ELISA kit incorporates antibodies highly specific for macrocyclic

trichothecenes immobilized on polystyrene microtiter wells (4). Successful applications have been previously described (2, 3, 19). All reagents and antibody-coated wells were allowed to equilibrate to room temperature before use. For testing, samples or control mixtures were added to wells in triplicate. Following the incubation, wells were read at 450 nm using an EL-312 microtiter plate reader (Bio-Tek Instruments, Winooski, VT).

ELISA Interpretation

Data were expressed as percent inhibitions and relative trichothecene concentrations. Percent inhibitions were derived from raw data and were based on patient samples versus NHS controls run in parallel. The percent inhibition represents the degree of inhibition the test sample had on the capability of the satratoxin G-HRP conjugate to bind to the immobilized antibody. They were calculated as done by Schick et al., (39) using the following equation:

$$\% \text{ Inhibition} = 100 \times 1 \quad [(O.D._{450} \text{ sample} - \text{background})/(O.D._{450} \text{ control-background})]$$

To obtain relative trichothecene concentrations, an ELISA-based macrocyclic trichothecene standard curve was developed by testing a mixture of four macrocyclic trichothecenes (satratoxins G and H, verrucarin A, and roridin A) in equal amounts. Satratoxins G and H were purified as described by Hinkley et al. (21) in our laboratory. Roridin A and verrucarin A were purchased from Sigma (St. Louis, MO). Dilutions were made in NHS from a concentrated stock solution of the toxins in methanol (250 μg of each toxin) resulting in 12 test concentrations: 500, 250, 100, 50, 25, 10, 5, 2.5, 1, 0.5,

0.25, and 0.1 ng/ml. Each sample was extracted with acetonitrile as already described and tested using the ELISA. Average ELISA absorbances (from three replicates) at 450 nm were plotted against calculated toxin concentrations to generate a standard curve. Using this curve, an approximate trichothecene amount was determined for each sample in ng/ml.

Statistical Analysis

Statistical analyses were performed on serum samples using Sigma Stat 3.0 software (Chicago, IL). Mean OD_{450}s of individual test samples were compared to NHS using either a Student's *t*-test or one-way analysis of variance (ANOVA). All requirements for normality and equal variance were met for these analyses. Statistical analysis was also performed between Groups 1-3 using a Kruskal-Wallis one-way ANOVA as normality requirements could not be met. Statistical significance for all analyses was reported as having a P-value of less than 0.05.

Mass Spectrometry Analysis

Mass spectrometry analysis was performed on Samples 1 and 2 (because of their high trichothecene concentrations; see Results) using a Sciex API 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA). A 150 mm x 4.6 mm Zorbax SB-C8 (5 micron particle size) analytical column was used for the analyses. The flow rate was set for 0.6 ml/min with an injection volume of 20 µl. Acetonitrile-extracted serum samples were lyophilized and reconstituted in 1 ml of methanol. The samples were sonicated and vortexed for approximately five minutes prior to aliquoting into a 96 well polypropylene

sample block. Calibration standards (roridins A, E, H, and L-2, verrucarins A and J, satratoxins G and H, and isosatratoxin F) were kindly provided by Dr. Bruce B. Jarvis of the University of Maryland. These standards were prepared at concentrations ranging from 0.100 to 100 ppb in methanol. Samples (standards, test serum samples, and a roridin A spiked NHS sample) were injected directly onto the LC/MS/MS system. They were run in an aqueous mobile phase in which the gradient changed from 80% acetonitrile to 90% of a 25 mM ammonium acetate, 0.5% acetic acid solution in 16 minutes. Samples were analyzed in multiple reaction monitoring (MRM) MS/MS mode after which a full scan analysis via electrospray ionization (at 550°C) in positive ion mode was performed. Following the full scan, a precursor ion scan analysis was performed on the serum samples. With precursor ion scan analysis, molecular masses that arise from a common fragment ion could be derived. The logic for this approach was that, because trichothecene mycotoxins are analogs of each other, a common fragment would exist.

Results

Trichothecene Mycotoxin Analysis

Average ELISA percent inhibitions and relative trichothecene concentrations for serum samples in Groups 1 and 2 are shown in Tables 4.1 and 4.2, respectively. The standard curve used for calculated trichothecene concentrations is shown in Figure 4.1. More positives were seen in Group 1 individuals with known *Stachybotrys* exposure than those individuals in Group 2 (77.7% versus 34.6%). The majority of calculated trichothecene concentrations were low, averaging below or just above the set limit of

detection (0.1 ng/ml). Samples 1 and 2 from Group 1 demonstrated uniquely high concentrations (42.8 and 83.6 ng/ml, respectively). Table 4.3 shows the results obtained with Group 3 (negative controls). Of these, only NC 2 demonstrated a significant ($P<0.05$) positive response in the ELISA, representing 3.8% of the samples. Trichothecene concentrations for the majority of the negative controls fell below 0.1 ng/ml. Overall, there was a significant difference ($P<0.05$) between tests (Groups 1 and 2) and controls (Group 3). Individually, Group 1 was significantly different than Groups 2 and 3, but Groups 2 and 3 were not significantly different from each other. Median percent inhibitions for Groups 1, 2, and 3 were 16.3, 5.8, and 2.6%, respectively. Even when the high responders were removed from Group 1, statistical significance remained unchanged.

Mass Spectrometry Analysis

No detectable compounds (based on standards) were present after MRM analysis in the experimental serum samples. Limits of detection for the standards were 0.100 ng/ml for the roridins and verrucarins, 1.00 ng/ml for isosatratoxin F, 2.00 ng/ml for satratoxin H, and 10.0 ng/ml for satratoxin G. Roridin A was positively identified in the spiked control sample demonstrating that the cleanup methods were successful in extracting the trichothecene mycotoxins. A full scan analysis of the experimental serum samples between m/z 155-700 atomic mass units (amu) generated several significant peaks. One peak of interest at a mass of 414 had similar spectral qualities at the trichothecene standards (i.e. a similar adduct pattern). Precursor ion scan analysis resulted in the discovery of two common fragments with precursor masses of 440 and

484. The 484 mass was the expected mass of verrucarin J, but had a different retention time on the column than the purified standard. These masses were unique to the test serum samples and were not detected in the roridin A-spiked NHS positive control sample.

Discussion

In this study, we were successful in demonstrating the presence of trichothecene mycotoxins in serum samples from individuals exposed to mold (primarily *Stachybotrys*) in water-damaged indoor environments. Our findings indicate that these highly toxic compounds can actually be found in people exposed to these environments and therefore have the potential to negatively affect the health of such individuals. This relationship is further strengthened by our previous investigations which demonstrated that *Stachybotrys* trichothecene mycotoxins become airborne and could be detected in buildings contaminated with this organism (2, 3).

Through the use of an ELISA that incorporates a macrocyclic trichothecene-specific polyclonal antibody, we were able to detect and quantify trichothecene mycotoxins in the serum samples. Most of the experimental samples demonstrated low percent inhibitions and trichothecene concentrations versus negative and NHS controls (Tables 1-3). One possible reason for this is that trichothecene concentrations in the buildings where the individuals lived/worked were unknown and untested. Concentrations of fungi were known but this does not accurately assess mycotoxin exposure (44). We have shown that trichothecene levels in mold-contaminated building are relatively low (2, 3). It might, therefore, be expected that concentrations in naturally

exposed humans would be even lower (possibly below the limit of detection of current technology). This is not to say that such low levels do not pose a human health risk, particularly as it has been shown that these mycotoxins act at very low concentrations (30, 49).

Many studies have shown that non-macrocyclic trichothecenes such as T-2 toxin, verrucarol, nivalenol, and deoxynivalenol are rapidly metabolized in animal models (1, 5, 20, 34-36). It is therefore possible that the trichothecenes for which we were testing were broken down in a rapid fashion prior to our analyses. To our knowledge, metabolic studies have not been conducted concerning the macrocyclic trichothecenes produced by *Stachybotrys chartarum*. Being that all trichothecene mycotoxins are structurally related compounds, it is reasonable to believe that the macrocyclics would be metabolized in a similar fashion. The likelihood that these toxins are bioaccumulated in the human body and modify the immune response is another possibility. In fact, aflatoxin-albumin adducts were detected in 93% of Gambian children exposed to contaminated staple foods (46). Moreover, due to the binding of mycotoxins to human serum proteins, antibodies against macrocyclic trichothecene mycotoxins have been reported in blood samples of patients with repeated exposure to fungi in water-damaged buildings (43, 47, 48). In these studies it was concluded that detection of antibodies indicates exposure to a substance and may provide supporting evidence for a specific etiologic exposure. Furthermore, for the present studies, not only was the relative concentration of trichothecenes significantly high in 77.7% of the individuals with known *Stachybotrys* exposure (Group 1), but when antibodies against trichothecene bound to human serum albumin were measured in a set of these individuals, a 3- to 15-fold increase (versus

controls) in these antibody levels was seen (47). The possible bioaccumulation of these mycotoxins is also supported by the fact that some mycotoxins such as ochratoxin A and the aflatoxins are fairly long-lived and have been detected in animal tissue or blood products many weeks after exposure (24).

Two of the serum samples demonstrated uniquely high trichothecene concentrations and were analyzed further by LC/MS/MS. Results showed the presence of two trichothecene-like compounds, but neither could be positively identified based on nine purified standards. Because these samples demonstrated spectral qualities similar to the purified trichothecenes (related adduct patterns) and demonstrated high ELISA reactivity, it is highly likely that trichothecenes were present. One of the isolated compounds had a molecular mass of 484 which is the mass of verrucarin J, a macrocyclic trichothecene produced by *S. chartarum*. However, on the column, it had a different retention time than verrucarin J indicating that it may have been a positional isomer. There are several possible reasons why we could not make a positive identification. The simplest explanation is that we did not have the proper standards for comparison. Based on the high ELISA reactivity, we analyzed the serum samples for the presence of intact macrocyclic trichothecene mycotoxins as non-macrocyclic trichothecenes do not impart a high degree of reactivity in the assay used (2, 4). It is possible that we detected an uncharacterized macrocyclic trichothecene or unknown metabolic breakdown product that still conferred positive ELISA reactivity. Because of the cleanup/extraction method used and the specificity of the ELISA, it is unlikely that we were detecting false positives. This is particularly true for Samples 1 and 2 of Group 1.

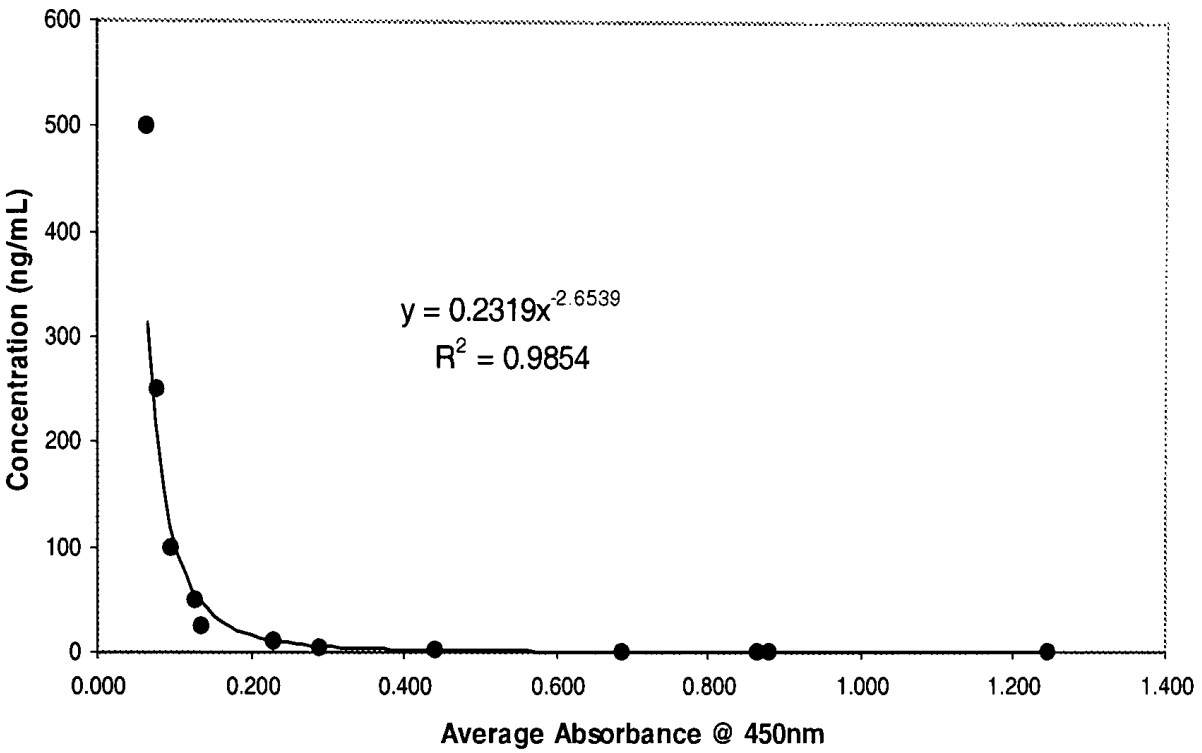
One of the negative controls (NC 2) was significantly different than NHS when tested. This particular control was re-tested several times and similar results were seen for each evaluation. Serum samples used as negative controls were randomly selected from clinical samples and were categorized as being from individuals with unknown degrees of mold and/or mycotoxin exposure. It is therefore possible that the particular individual from whom NC 2 was collected had been or was currently being exposed (i.e., living or working in a contaminated environment) to *Stachybotrys* and its associated trichothecene mycotoxins.

In conclusion, we have been able to show that trichothecenes can be extracted and detected in human sera from individuals who have been exposed to *Stachybotrys* in contaminated indoor environments. Our data stress the need for further study in this area. Specifically, there is a great requirement to understand the distribution and metabolic processes that take place following inhalation of *S. chartarum* mycotoxins. To date, this has not been examined *in vivo*. Controlled animal studies could clarify these types of issues and offer a better understanding into the health risks associated with exposure to *Stachybotrys* and its associated trichothecene mycotoxins in contaminated buildings. Furthermore, the studies presented here could be expanded upon and more strictly controlled. This could consist of extensive documentation of control and test samples including extent of *Stachybotrys* growth and airborne trichothecene concentrations in the buildings, time of occupant exposure when the serum was drawn, symptomology and physical examinations on a per patient basis. Detailed physical examinations were provided with some of the samples and a correlation was seen between more severe symptoms (nausea, vomiting, diarrhea, epistaxis, etc.) and the presence of trichothecenes,

but such reports were not provided with all samples and a significant relationship could not be made. Collaboratively, future studies could further clarify the relationship between the presence of mold and mycotoxins and adverse human health effects.

Figure 4.1. ELISA Based Macrocyclic Trichothecene Standard Curve Derived from Spiked Normal Human Serum Extracts. Satratoxins G and H, roridin A, and verrucarin A were mixed in equal concentrations in methanol, diluted in PBS (500 to 0.1 ng/ml), and used to spike normal human serum. These samples were then subjected to the extraction procedure outlined in the text. Samples were tested using a macrocyclic trichothecene-specific ELISA. OD_{450}s were plotted against toxin concentrations as the power curve shown here. This standard curve was used to estimate macrocyclic trichothecene equivalents from experimental filter extracts. For reference, the trichothecene concentrations, OD_{450}s, and percent inhibitions are shown in a table format below the graph. Standard deviations (representing three replicates) for all values are noted on the graph and in the table.

Macrocyclic Trichothecene Standard Curve Based on Acetonitrile Extracts of Normal Human Serum Spiked with Roridin A, Verrucarin A, and Satratoxins G & H - Average Concentrations Based on ELISA (500 to 0.1 ng/mL)



Concentration (ng/ml)	Avg OD_{450}	Avg % Inhibition
500	0.07 ± 0.003	95.9 ± 0.2
250	0.08 ± 0.002	95.2 ± 0.1
100	0.10 ± 0.004	94.0 ± 0.2
50	0.13 ± 0.002	92.0 ± 0.1
25	0.14 ± 0.01	91.4 ± 0.4
10	0.23 ± 0.003	85.5 ± 0.2
5	0.29 ± 0.02	81.9 ± 1.5
2.5	0.44 ± 0.01	72.3 ± 0.3
1	0.69 ± 0.05	57.0 ± 3.2
0.5	0.86 ± 0.04	45.9 ± 2.4
0.25	0.88 ± 0.14	44.9 ± 8.9
0.1	1.25 ± 0.03	21.8 ± 1.8

Table 4.1. Competitive ELISA results of Group 1 serum samples - average percent inhibitions and relative trichothecene concentrations.

Sample[a]	Average Percent Inhibition[b]	Relative Trichothecene Concentration (ng/ml)[c]
1*	90.8 ± 0.1	42.8 ± 1.1
2*	92.8 ± 0.3	83.6 ± 9.2
3*	18.2 ± 2.1	0.13 ± 0.01
4	0.0 ± 0.0	NA
5	11.1 ± 8.9	0.11 ± 0.03
6*	17.6 ± 2.4	0.13 ± 0.01
7	8.5 ± 1.5	0.10 ± 0.004
8*	22.0 ± 1.1	0.10 ± 0.004
9	4.2 ± 3.4	BLD (0.09 ± 0.01)
10*	24.6 ± 10.3	0.17 ± 0.05
11*	14.0 ± 5.5	0.12 ± 0.02
12*	13.7 ± 1.4	BLD (0.07 ± 0.003)
13*	14.2 ± 4.5	BLD (0.07 ± 0.01)
14*	14.3 ± 1.4	BLD (0.07 ± 0.003)
15*	18.0 ± 0.6	BLD (0.08 ± 0.002)
16*	21.2 ± 9.4	0.10 ± 0.03
17*	13.3 ± 1.6	BLD (0.07 ± 0.003)
18*	20.9 ± 3.5	0.15 ± 0.02

[a]Samples indicated with an * represent those samples that were significantly different than control (NHS) when the ELISA was performed.

[b]Percent inhibitions (± standard deviations). Values were based on results obtained using NHS when the ELISA was performed. Values represent triplicate wells.

[c]Trichothecene concentrations (± standard deviations). Values were obtained using the trichothecene standard curve shown in Figure 4.1. Results indicated with BLD (below limit of detection) represent those data that fell below the detection limit set on the standard curve. Extrapolated values are shown in parentheses. NA; not applicable

Table 4.2. Competitive ELISA results of Group 2 serum samples - average percent inhibitions and relative trichothecene concentrations.

Sample[a]	Average Percent Inhibition[b]	Relative Trichothecene Concentration (ng/ml)[c]
19	0.31 ± 0.53	BLD (0.06 ± 0.01)
20	0.0 ± 0.0	NA
21	0.0 ± 0.0	NA
22	0.0 ± 0.0	NA
23	7.8 ± 0.9	0.12 ± 0.003
24	7.6 ± 4.6	0.10 ± 0.01
25	5.2 ± 5.8	BLD (0.09 ± 0.02)
26	3.5 ± 3.1	BLD (0.09 ± 0.01)
27	0.44 ± 0.76	BLD (0.08 ± 0.01)
28*	8.0 ± 0.3	0.11 ± 0.001
29	2.9 ± 3.8	BLD (0.09 ± 0.01)
30	4.7 ± 4.3	0.10 ± 0.01
31	1.5 ± 2.1	BLD (0.09 ± 0.01)
32*	10.0 ± 3.4	0.12 ± 0.01
33	3.8 ± 1.3	0.10 ± 0.003
34*	14.7 ± 6.7	BLD (0.08 ± 0.02)
35	8.8 ± 3.9	BLD (0.06 ± 0.07)
36	2.9 ± 2.8	BLD (0.05 ± 0.004)
37*	20.9 ± 8.7	0.10 ± 0.03
38*	21.6 ± 9.4	0.10 ± 0.04
39	4.0 ± 1.8	BLD (0.06 ± 0.003)
40*	8.8 ± 2.6	BLD (0.06 ± 0.01)
41	4.0 ± 4.9	BLD (0.06 ± 0.01)
42*	8.9 ± 1.1	BLD (0.06 ± 0.002)
43*	10.4 ± 0.5	BLD (0.07 ± 0.001)
44*	13.6 ± 3.8	BLD (0.07 ± 0.01)

[a]Samples indicated with an * represent those samples that were significantly different than control (NHS) when the ELISA was performed.

[b]Percent inhibitions (± standard deviations). Values were based on results obtained using NHS when the ELISA was performed. Values represent triplicate wells.

[c]Trichothecene concentrations (± standard deviations). Values were obtained using the trichothecene standard curve shown in Figure 4.1. Results indicated with BLD (below limit of detection) represent those data that fell below the detection limit set on the standard curve. Extrapolated values are shown in parentheses. NA; not applicable

Table 4.3. Competitive ELISA results of Group 3 serum samples - average percent inhibitions and relative trichothecene concentrations.

Sample[a]	Average Percent Inhibition[b]	Relative Trichothecene Concentration (ng/ml)[c]
NC 1	0.0 ± 0.0	NA
NC 2*	15.0 ± 3.4	BLD (0.08 ± 0.008)
NC 3	5.6 ± 2.6	0.11 ± 0.01
NC 4	5.4 ± 2.2	0.11 ± 0.01
NC 5	4.0 ± 2.1	0.10 ± 0.01
NC 6	9.8 ± 3.2	BLD (0.09 ± 0.01)
NC 7	2.1 ± 2.0	0.10 ± 0.01
NC 8	1.9 ± 2.8	BLD (0.05 ± 0.01)
NC 9	4.8 ± 4.8	0.11 ± 0.02
NC 10	5.3 ± 2.0	0.11 ± 0.01
NC 11	0.0 ± 0.0	NA
NC 12	1.4 ± 2.3	BLD (0.08 ± 0.01)
NC 13	4.2 ± 3.7	BLD (0.09 ± 0.01)
NC 14	0.0 ± 0.0	NA
NC 15	2.3 ± 3.3	BLD (0.09 ± 0.01)
NC 16	1.1 ± 2.0	BLD (0.09 ± 0.01)
NC 17	0.25 ± 0.33	BLD (0.09 ± 0.002)
NC 18	0.74 ± 0.80	BLD (0.09 ± 0.01)
NC 19	0.71 ± 1.2	BLD (0.08 ± 0.01)
NC 20	4.9 ± 2.7	BLD (0.06 ± 0.004)
NC 21	10.1 ± 4.9	BLD (0.07 ± 0.01)
NC 22	2.1 ± 3.6	BLD (0.05 ± 0.01)
NC 23	6.6 ± 6.8	BLD (0.08 ± 0.01)
NC 24	3.3 ± 1.2	BLD (0.05 ± 0.002)
NC 25	1.3 ± 1.3	0.10 ± 0.01
NC 26	5.1 ± 4.2	BLD (0.06 ± 0.01)

[a]Samples indicated with an * represent those samples that were significantly different than control (NHS) when the ELISA was performed.

[b]Percent inhibitions (± standard deviations). Values were based on results obtained using NHS when the ELISA was performed. Values represent triplicate wells.

[c]Trichothecene concentrations (± standard deviations). Values were obtained using the trichothecene standard curve shown in Figure 4.1. Results indicated with BLD (below limit of detection) represent those data that fell below the detection limit set on the standard curve. Extrapolated values are shown in parentheses. NA; not applicable

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CHAPTER V

DISCUSSION

Very few subjects have been as scientifically elusive as the relationship between the presence of fungi in the indoor environment and adverse human health effects resulting from such exposures. It is well accepted that the overall quality of the air in environments where people work and live has a major influence on health. However, indoor air is extremely complex in nature, being composed of a seemingly endless list of compounds and particulate matter, of which no one factor can be attributed to the problem. Additionally, unlike controlled laboratory situations, no two indoor environments are alike. Taken together, these issues have resulted in the establishment of a confusing, and often conflicting, field of study. Though it is impossible to pinpoint one cause for the numerous reported health complaints associated with poor indoor air quality, the presence of *Stachybotrys chartarum* and its associated trichothecene mycotoxins has been hypothesized to be a significant contributor to such issues. Currently, very few quantitative data exist regarding this relationship. Therefore, we approached this project with the goal to better understand the roles that *S. chartarum* and trichothecene mycotoxins may play in indoor air quality and human health.

The types of fungi that have been isolated from indoor environments number into the hundreds. A small percentage of these (< 10%) are commonly isolated from water-damaged buildings. Of these, very few are thought to be able to significantly influence human health in the context of indoor environments. To further narrow this field, *Stachybotrys chartarum* and *Chaetomium globosum* are perhaps the only indoor fungi

capable of producing compounds toxic enough to result in such often reported symptoms as nausea, diarrhea, dizziness, hemorrhage, and varying degrees of neurological damage. *C. globosum* is frequently isolated from water-damaged buildings, but studies involving its significance in indoor environments are currently in their infancy. *C. globosum* is known to produce mycotoxins that inhibit actin polymerization and, consequently, eukaryotic cell division (24, 33, 37), but it is not known what role this organism and its associated mycotoxins play in contaminated buildings. Conversely, *S. chartarum* and associated trichothecene mycotoxins have been shown to adversely affect animal and human health in many instances (8, 9, 12, 13, 15, 19). In fact, many of the symptoms currently reported by occupants of *S. chartarum*-contaminated buildings are no different than those initially described in animals and individuals who came in close contact with this organism over 70 years ago. Because of its historical significance and well-documented toxicity, *S. chartarum* was viewed as the organism with the most impact on human health in indoor environments and thus the focus of our research.

The first phase of our research was devoted to demonstrating that *S. chartarum* trichothecene mycotoxins can and do become airborne. A caveat of this was that these mycotoxins were associated with highly respirable particulate matter, most notably particles that were smaller than intact conidia. This approach was important for two main reasons. First, regarding indoor environments, the primary route of exposure to these mycotoxins is inhalation. Other routes of exposure have been studied in humans, but are not applicable toward indoor environments. Direct contact with *S. chartarum* was shown to produce blistering, inflamed efflorescences and scaling of the skin (11, 40), but not the severe symptoms often reported in *S. chartarum*-contaminated buildings. Controlled

intravenous administration of diacetoxyscirpenol, a non-macrocyclic trichothecene, was examined in cancer patients and resulted in symptoms such as nausea, vomiting, lethargy, and confusion. Though symptoms were similar to those frequently reported by occupants of contaminated indoor environments, intravenous exposure would never occur in a building situation.

Another reasoning behind our approach revolved around the fact that airborne concentrations of *Stachybotrys* conidia in most contaminated buildings are low or undetectable, even with visible surface contamination (personal communication, Center for Indoor Air Research, Texas Tech University Health Sciences Center). This is primarily due to their large size in conjunction with their "sticky" properties that result in the formation of large conidia clumps that possess poor aerodynamic properties and therefore do not remain airborne for long periods of time. Though conidia can be detected, they are generally in quantities considered too low to pose a significant human health risk alone. The poor respirable properties of *Stachybotrys* conidia and their overall low airborne abundance in contaminated indoor environments led us to question how occupants of these buildings exhibit ailments resembling exposure to trichothecene mycotoxins. It has been well established that macrocyclic trichothecene mycotoxins are associated with *S. chartarum* conidia (i.e., the toxins are not secreted into the environment). We therefore developed the hypothesis that airborne *S. chartarum* trichothecene mycotoxins could exist free from intact conidia on highly respirable particles such as fungal fragments and/or various types of dust and debris. Pasanen et al., (26) showed that *Stachybotrys* toxins were concentrated in airborne fungal propagules (conidia) and were not present as free aerosols in the air. Furthermore, because *S.*

chartarum is sensitive to dessication (3), concentrations of airborne fragments can potentially far exceed those of conidia. These ideas were supported by several studies that demonstrated the existence and importance of this class of particles (16, 22, 23, 28).

To test our first hypothesis, we designed a controlled air sampling setup whereby we generated particulate matter from *S. chartarum* grown on ceiling tile (Figure 2.1) and rice (Figure B.1) and collected these particles based on their size. For detection purposes, we used an ELISA that incorporates a polyclonal antibody with a high specificity for macrocyclic trichothecenes. Cross-reactivity to non-macrocyclic trichothecenes was minimal and no significant cross-reactivity was seen to other classes of mycotoxins. Currently, the most common and accepted methods for mycotoxin analysis include thin-layer, liquid, and gas chromatography, as well as mass spectrometry. Although very powerful tools, a major impediment to these approaches is the overwhelming interferences found in samples such as indoor air (27). This "noise" can essentially mask any compounds of interest, particular if they exist in limiting quantities like the trichothecenes produced by *S. chartarum*. Analysis of most samples via these techniques requires a series of extensive cleanup steps to overcome the interferences. These procedures are often time-consuming and expensive and cannot ensure the complete removal of these interfering compounds. Assuming that airborne concentrations of trichothecene mycotoxins are low, even minimal loss of sample is undesirable. The use of immunoassays for the detection and quantification of trichothecene mycotoxins has been shown to be a sensitive and specific means for analysis of these compounds (5-7, 10, 27). The ELISA used for our studies had many advantages and proved to be a valuable and effective tool. For confirmatory purposes, we also incorporated the use of

HPLC and SEM. Using HPLC, airborne macrocyclic trichothecenes (satratoxin H) were positively identified but it could not be confirmed that they were on particulates smaller than conidia. We attribute this to the difficulty of particle separation. As stated by Sorenson et al., "Total separation of conidia from hyphal fragments and other particles [is] not possible," (35). Although not a trivial task (as expressed by these authors), we showed that separation was possible and presented a feasible mechanism for how *S. chartarum* trichothecene mycotoxins are released into the air and consequently inhaled by occupants of "sick" buildings. In fact, we contend that the majority of the collected and detected trichothecene mycotoxins were present on particles free from and smaller than conidia as very few intact conidia and larger particles were seen on any of the filters, regardless of pore size.

The second phase of our research focused on determining if airborne *S. chartarum* trichothecene mycotoxins were present in natural situations (i.e., indoor environments contaminated with this organism). In our attempts to understand mycotoxin production by *Stachybotrys*, we discovered that trichothecene production by this fungus is relatively low, especially when grown on building materials [data not shown]. Our initial HPLC analyses required large amounts of starting material (greater than 1 kg of *Stachybotrys*-contaminated rice) to begin characterizing trichothecene production by this organism. To support these findings, Rand et al. estimated that one *Stachybotrys* conidium contains approximately 1 picogram of satratoxin G equivalents (29). Based on these observations, we formed the hypothesis that airborne trichothecene concentrations in contaminated indoor environments were extremely low and difficult to detect. The macrocyclic

trichothecene-specific ELISA was a suitable method of detection, but finding a means of collection was a difficult task.

Most currently available methods for sampling indoor environments are not suitable for trapping and/or collecting limited amounts of small molecular weight chemicals such as the trichothecenes. Impaction samplers are designed to assess the viability and types and of microorganisms present in the air. Other commonly used air sampling methodologies include filtration and liquid impingement devices that sample with flow rates insufficient to collect and analyze for the presence of trichothecenes. These devices are efficient screening tools, but are not designed for collecting airborne mycotoxins. To prove our hypothesis, a means to collect large sample sizes was necessary. Commercially available air purifiers incorporating fibrous filter material were initially used for sampling contaminated environments. These devices sampled high volumes of air (greater than 500 LPM), but resulted in undesirably high background noise and false positives on ELISA testing. This was attributed to error-prone sample workup procedures that resulted in the extraction of unwanted compounds from the filter material alone [data not shown]. For the current studies, the SpinCon PAS 450-10 Bioaerosol Sampler and the Andersen PUF High Volume Air Sampler were chosen as the testing devices. The SpinCon sampler was originally designed to collect a wide range of potentially hazardous chemical and biological agents, specifically those considered as threat agents (i.e., sarin gas or *Bacillus anthracis* spores). The PUF sampler was intended for testing airborne pesticide concentrations in outdoor environments. Being controlled high volume air sampling devices, the use of these systems as indoor air samplers for our purposes was promising. The SpinCon was used for the majority of the testing due to its

portability and ease of use. The PUF sampler was modified to separate and collect particles of differing sizes on membrane filters as done in our first set of experiments.

Eight buildings with varying degrees of water damage and mold contamination throughout the state of Texas were chosen for sampling. Air sampling was performed under static and mechanically disturbed conditions. Air disturbance was initiated as an attempt to resemble what may occur under normal circumstances. Previous studies have suggested and demonstrated that even subtle air disturbance mechanisms can drastically affect (increase) the amount of airborne particulate matter (4, 11, 22). Our results showed that airborne trichothecene mycotoxins were present in buildings contaminated with *S. chartarum* and that air disturbance increased the amount of these compounds. Furthermore, in one building, we were able to repeatedly separate and collect these toxins on particulates smaller than conidia.

The final aim of our research was to determine if trichothecene mycotoxins could be detected in individuals exposed to *S. chartarum*-contaminated buildings. Dr. Bruce B. Jarvis, a leader in the field of *Stachybotrys* mycotoxin chemistry, recently expressed that one of the major problems in assessing human exposure to indoor mycotoxins has been the absence of a reliable means for measuring the level of exposure (20). Our first two studies demonstrated that trichothecene mycotoxins from *S. chartarum* can become airborne and exist in detectable amounts in contaminated indoor environments. However, this does not directly correlate to human exposure. Biomarkers for mycotoxins in humans, which may include the toxin itself or a well-characterized metabolite, have been described in very few studies (17, 25, 32, 38). For the vast majority of mycotoxins, these characterized biomarkers have not been discovered. Moreover, information on the

pharmacokinetics of these compounds in animals, much less humans, is virtually unknown. This is especially true for trichothecene mycotoxins produced by *Stachybotrys*. Antibody production to *Stachybotrys* and its mycotoxins has been reported in individuals exposed to this organism (36, 39), but this type of measurement does not directly correlate to a current exposure. By detecting the actual mycotoxins in human sera, we have strengthened the cause and effect relationship between the presence of *Stachybotrys* and airborne trichothecenes in contaminated buildings and the potential to develop adverse health effects.

To bring the three phases of our research together, we present here a sequence of events and situations that we believe occur in "sick" buildings. To begin, in indoor situations where mold growth is a problem, there is always an initial water intrusion event. For the buildings we tested, these events were a result of poor and/or neglected construction, pipe leaks and condensation. Water damage is often followed by mold growth, particularly if the problem is not recognized soon after it arises. The constant temperatures and humidity in conjunction with large amounts of substrate (cellulose-containing building materials such as drywall, ceiling tile, and wood) present in indoor environments provide the perfect growth conditions for fungi. Although many fungi have the potential to grow indoors, one of the most commonly isolated organisms is *Stachybotrys chartarum*. We hypothesize this to be due to two factors. First, *S. chartarum* requires a high amount of water activity. Water damage in buildings is often unrecognized and/or neglected leading to a situation where the water activity of the building material (substrate) is equal to or greater than 90%. *S. chartarum* is one of the organisms capable of growing under such conditions. In hindsight, this is likely the

reason why we often find *Chaetomium globosum* growing together or in proximity to *Stachybotrys*, as it requires similar growth conditions. The second reason why *S. chartarum* is commonly found is that conidia from this fungus have been found inherently associated with the building material (21). This makes sense since building materials are mass produced and often stored in outdoor environments where *Stachybotrys* is naturally found (as a soil fungus). Additionally, building materials are not sterilized and are often not examined for prior water damage before being shipped for use in construction.

At some point after growth has been established, the colonies begin to dessicate due to the absence of a continual water influx. In many cases, the cause of the initial water damage is fixed, but the mold contamination remains. This is often because the presence of mold may not be realized in that the majority of growth may be hidden in inaccessible areas. As we showed with our first experiments, dessication leads to an increased amount of airborne particulates inclusive of trichothecene mycotoxins. Even though *Stachybotrys* growth may be hidden, occupant exposure is possible as particles have been shown to be able to penetrate through building structures (1). This is more apt to occur with highly respirable particles (less than 3 microns in diameter) such as those carrying trichothecene mycotoxins that we showed to originate from *S. chartarum* growth. Combined with air disturbance mechanisms, particles with associated mycotoxins are slowly released into the building air.

Rao et al. (31) concluded that "realistic exposures [to indoor mycotoxins] are probably chronic and at low concentrations." Though acute exposures are possible, the conclusions of Rao et al. are supported by our research that showed concentrations of

trichothecene mycotoxins in contaminated indoor environments vary depending on the amount of *Stachybotrys* growth and air disturbance, but are overall low. With airborne mycotoxins present on highly respirable particles, inhalation is inevitable. A plausible occupant exposure scenario can be extrapolated from our studies. We estimated airborne trichothecene levels ranging from approximately 4 to 250 picograms/m^3 of sampled air (median quantified concentrations). For example purposes, 100 picograms/m^3 of air will be assumed. With a minute ventilation of 6 LPM (under moderate activity), a human inhales approximately 0.36 m^3 of air per hour (34). In an environment with an airborne concentration of 100 picograms/m^3, an individual potentially inhales approximately 300 picograms of trichothecenes in an eight-hour period. The LD_{50} of satratoxin H in mice administered the toxin intravenously is 5.7 mg/kg body weight (30). Based on studies with T-2 toxin (40), it can be assumed that the LD_{50} value via the respiratory route is 10-fold less or approximately 600 μg/kg body weight for satratoxin H. With the above scenario, this concentration far exceeds what could possibly exist in contaminated buildings. However, when discussing exposure to *S. chartarum* trichothecene mycotoxins in indoor environments, immediate lethal doses are not an issue. Instead, the concentrations that may lead to the commonly reported symptoms of nausea, diarrhea, headaches, dizziness, hemorrhage, and others are of interest. Theoretically, these concentrations could be hundreds to thousands of times less than the reported LD_{50} values. Inhalation of 300 picograms per eight hours would result in approximately a 10 nanogram exposure over a period of a month. These concentrations certainly have the potential for the chronic development of adverse health effects in humans.

At low concentrations, trichothecenes are rapidly dissolved under physiological conditions (2, 18). We present evidence of this through our ability to collect airborne toxins in an aqueous solution (PBS) and the fact that they could be recovered in spiked and experimental human serum samples. Because of this property, we hypothesize that, following inhalation, trichothecenes are rapidly dispersed in the blood stream and begin to act upon cells and tissues with high rates of metabolism and protein synthesis. These include the lining of the digestive tract, bone marrow, spleen, liver, skin and hair follicles, and fetal tissue. Toxicity may also involve effects on DNA synthesis and cell membrane integrity (14). Metabolism likely occurs as indicated by our serum studies, but tissue damage may be relatively slow, assuming a chronic exposure.

The above scenario represents an ideal situation. Realistic exposure and ensuing health effects in contaminated indoor environments is likely a complicated set of events that is influenced by the presence of other molds, mycotoxins, allergens, and VOCs which may synergistically play a significant role. Additional factors such as age, immune status, smoking habits, etc. influence how *Stachybotrys* and associated trichothecene mycotoxins affect occupant health. Together, these issues have resulted in a complex field of study that cannot be easily generalized. Although we were able to consistently demonstrate the presence of trichothecene mycotoxins in the air of *Stachybotrys chartarum*-contaminated buildings and in the sera of exposed individuals, our results do not directly link the presence of *S. chartarum*/trichothecenes to human health. This relationship is currently speculative and will likely elude the scientific community for years to come.

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APPENDIX A
HIGH PERFORMANCE LIQUID CHROMATOGRAHPY ANALYSIS OF COMPOUNDS PRODUCED BY *Stachybotrys chartarum* WITH A FOCUS ON MACROCYCLIC TRICHOTHECENE CHARACTERIZATION

Background Information

For the HPLC analyses, *Stachybotrys chartarum*-contaminated rice was used for starting material. Trichothecene production was initially characterized on a variety of substrates including cellulose-containing ceiling tile, drywall, various types of media plates and rice. Toxin profiles were similar between the substrates, but fungal growth and ensuing trichothecene production was best (highest) on rice [data not shown]. Rice was therefore chosen for the analyses and purifications presented here.

The procedure for growing *S. chartarum* on rice was as follows: 250 grams of rice (Uncle Ben's) was added to 250 ml of water in a 1000 ml Erlenmeyer flask which was then autoclaved for 25 minutes. This was allowed to cool after which 25 ml of a *S. chartarum* conidia suspension (see Chapter II, Materials and Methods, Fungal Growth) was added. The flask was stoppered (Whatman BugStopper, Brentford, Middlesex, UK) and allowed to incubate for approximately 6 weeks at room temperature in sealed chambers supplied with separate, filtered air. To ensure proper distribution of the conidia throughout the rice, shaking (by hand) was performed every day for the first week followed by twice weekly for the next five weeks. This procedure resulted in a dark black rice culture that was ready for chemical extraction.

Development of a suitable extraction protocol was a trial and error process. Here we present the method that worked best for our needs. Rice demonstrating confluent *Stachybotrys* growth was extracted with HPLC grade methanol. Volumes varied and were based on amounts of starting material. The rice was extracted with three successive methanol washes that were pooled. This crude extract was allowed to evaporate to completeness (concentrate) under a fume hood at room temperature. The dried extract

was resuspended in a small volume of HPLC grade methanol and ultimately filtered through a nylon membrane filter with a 0.2 μm pore size. The filtered extract was then subjected to florosil column liquid chromatography (Fisherbrand PrepSep Solid-Phase Extraction Columns) for initial removal of unwanted compounds such as pigment and lipids. Columns were eluted with a 30% isopropanol/methylene chloride mixture which was then concentrated and resuspended as described above. This crude toxin extract was the final working solution for HPLC analysis.

HPLC analysis was performed as described throughout the text. Compound fractionation was performed using an Agilent 1100 Series Automatic Fraction Collector (model number G1364A). To achieve individual fractionation, the chromatography selectivity was changed by equipping the LC system with a 4.6 mm ID x 150 mm Zorbax RP-HPLC Bonded Phase column (Agilent Technologies). This allowed for a better separation of compounds that could not be established using our general methods (C8 column chromatography). Compounds were analyzed using Purify Software for Agilent Chemstations (Version A.01.01).

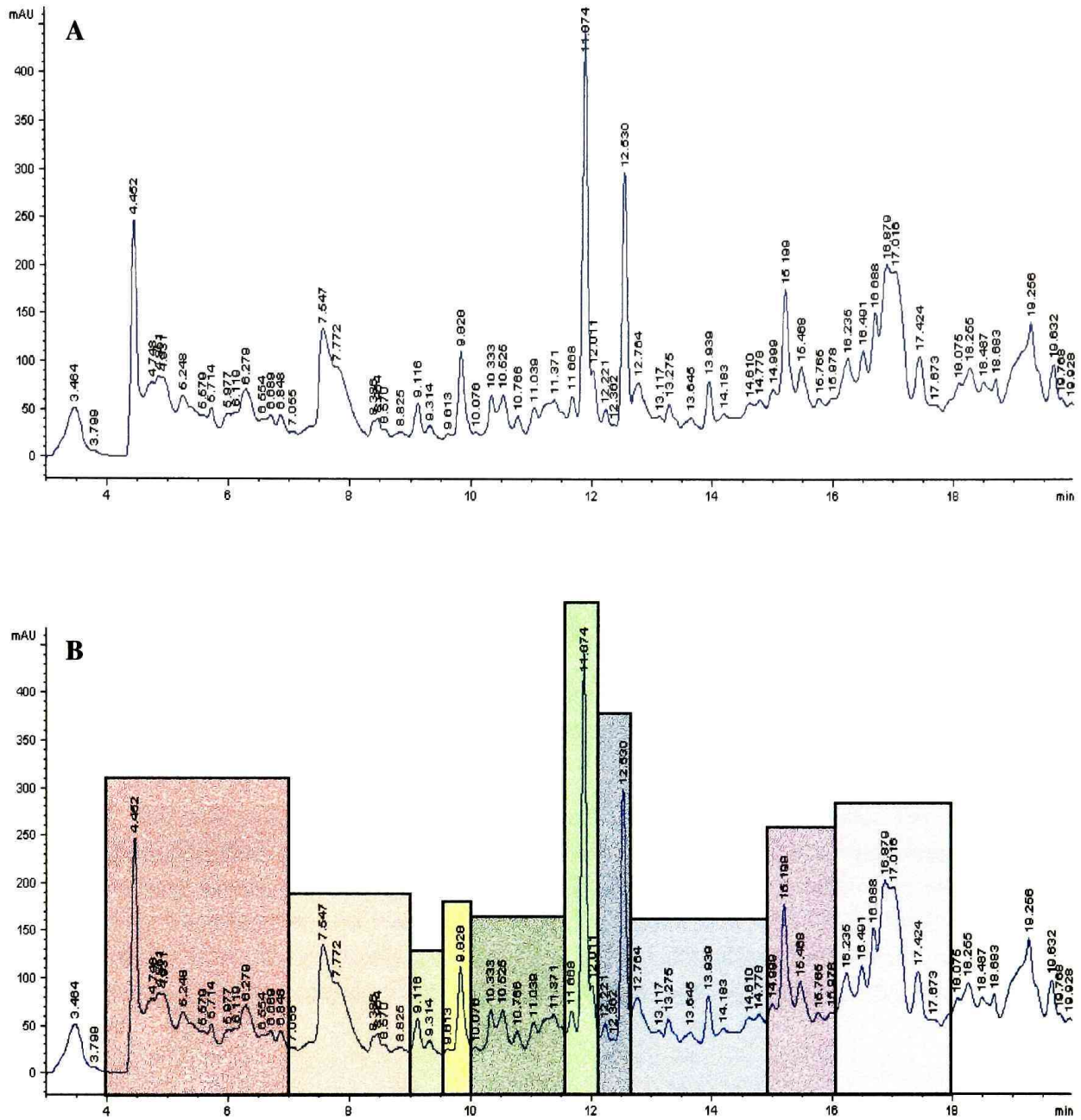


Figure A.1. HPLC Chromatograms of Crude *Stachybotrys chartarum* Extract and Initial 10 Fractions. Image A shows a representative chromatogram of crude *S. chartarum* extract. For all chromatograms, the x-axis is the retention time in minutes. Here, these times are noted above each individual peak. The y-axis is the relative absorbance at 260 nm of each compound in milli-absorbance units (mAUs). Over 90 compounds were separated in this run. Crude toxin extract was separated and collected into 10 individual fractions as shown in Panel B. Retention times were as follows: Fraction 1 (4.00-7.00 min); Fraction 2 (7.03-8.99 min); Fraction 3 (9.02-9.59 min); Fraction 4 (9.62-9.99 min); Fraction 5 (10.02-11.49 min); Fraction 6 (11.52-12.19 min); Fraction 7 (12.22-12.69 min); Fraction 8 (12.72-14.99 min); Fraction 9 (15.02-15.99 min); Fraction 10 (16.04-18.00 min).

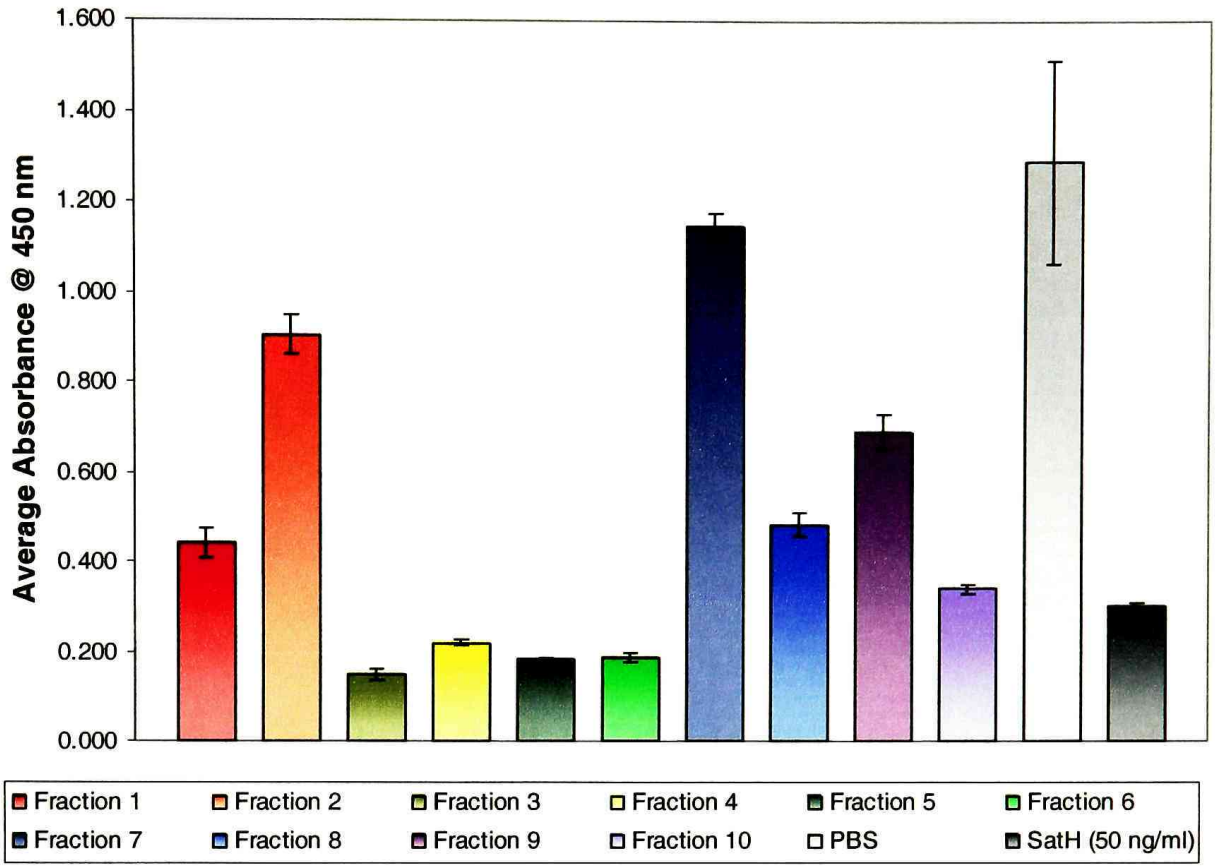


Figure A.2. Macrocyclic Trichothecene-Specific ELISA Results of Initial Ten Separated Fractions from *Stachybotrys chartarum* Crude Toxin Extract. HPLC-fractionated compounds were normalized to approximately 50 ng/ml (based on calibrated standards) in PBS. They were then tested via the macrocyclic trichothecene-specific ELISA as described in the text. Average absorbances (± standard deviations) at 450 nm are shown here. Fractions 3-6 demonstrated the highest responses and were chosen for further study (see below).

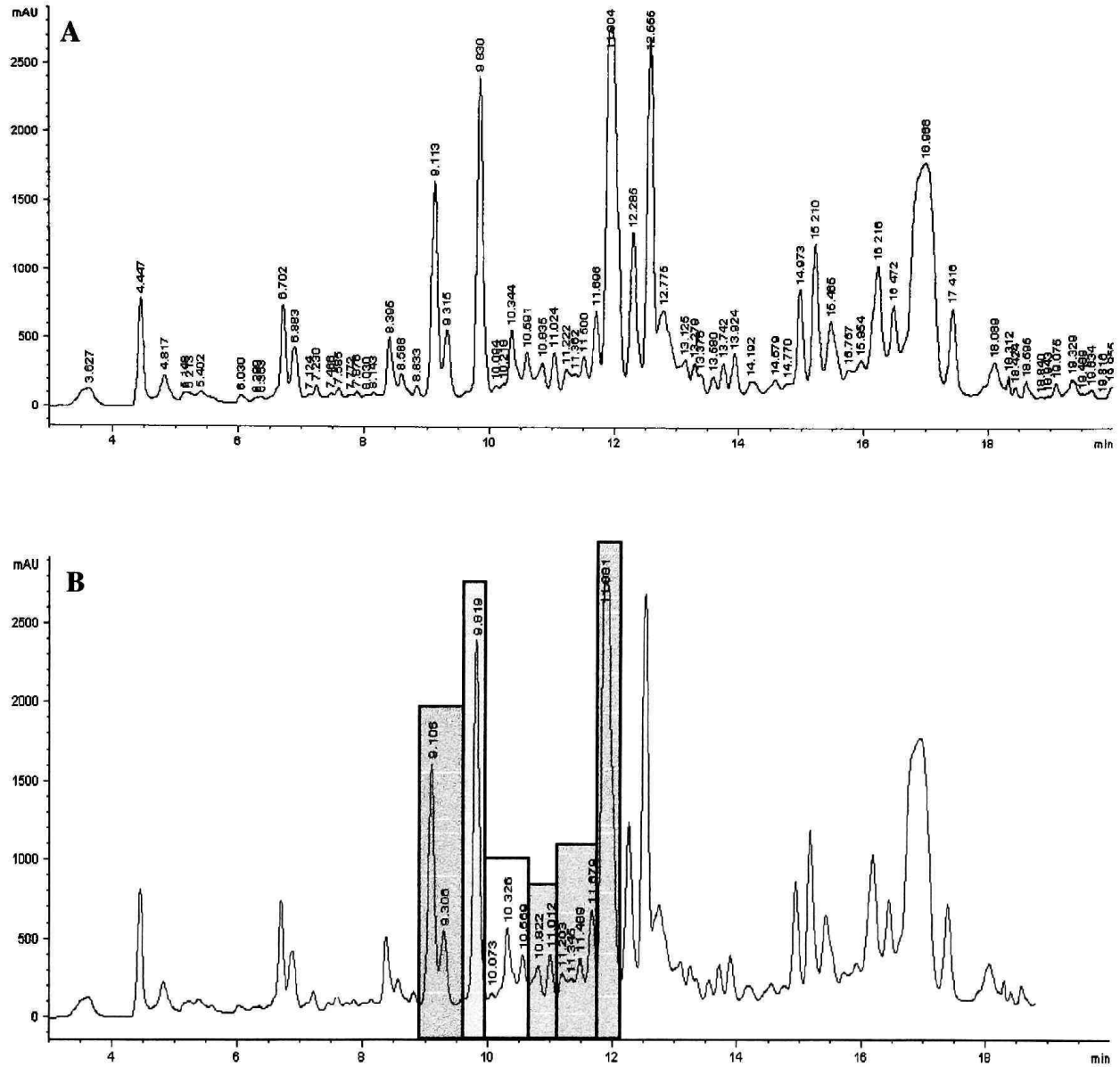


Figure A.3. HPLC Chromatograms of Crude *Stachybotrys chartarum* Extract and Further Characterized 6 Fractions. Image A shows a representative chromatogram of crude *S. chartarum* extract (different run than shown in Figure A.1). Based on ELISA analysis, compounds retaining on the column from approximately 9 to 12.1 minutes were chosen for further characterization. This range of compounds was separated and collected into the 6 fractions shown in Panel B.

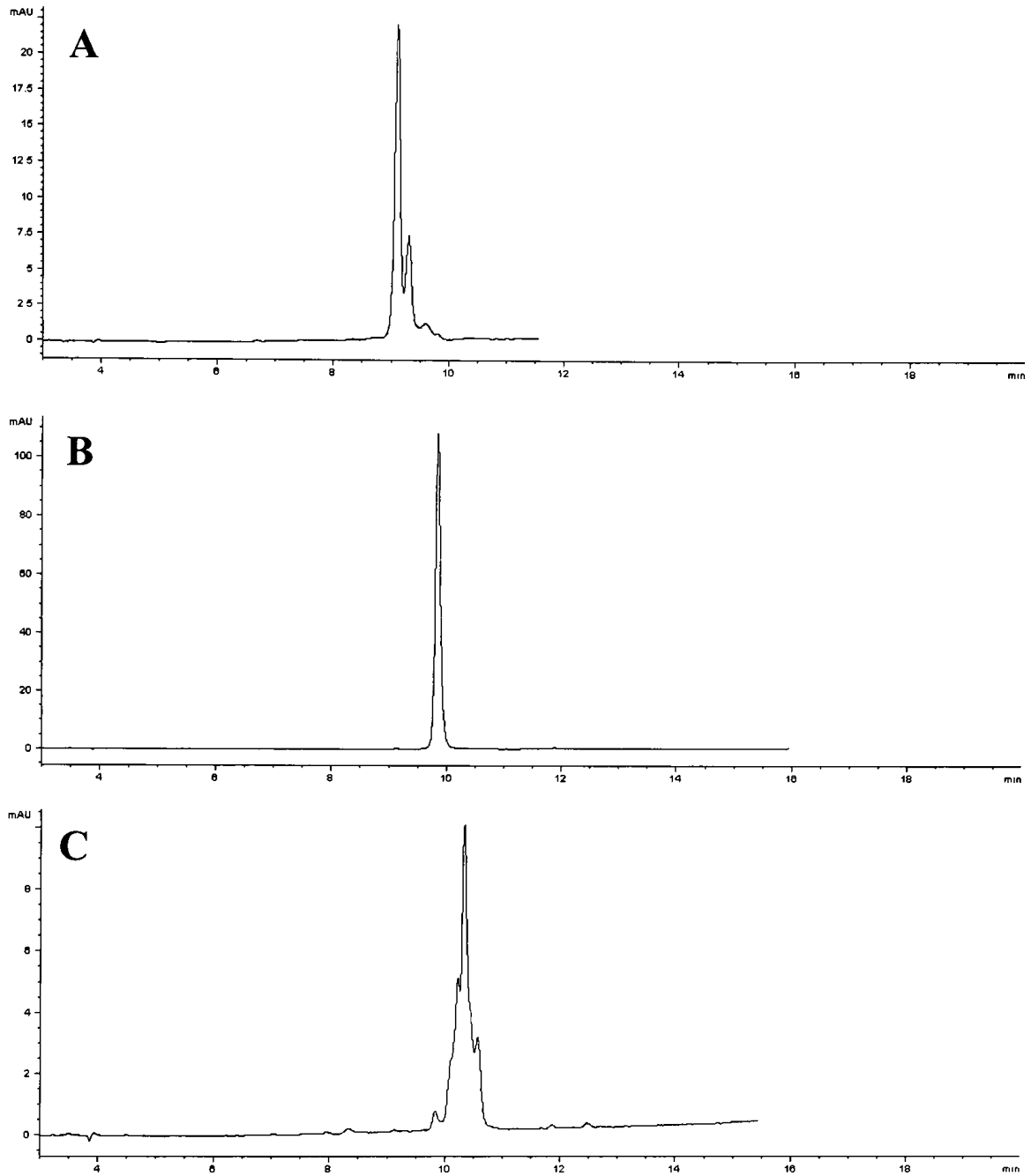


Figure A.4. HPLC Chromatograms of Fractions 1, 2, and 3. Retention times were as follows: Fraction 1 (8.90-9.70 min); Fraction 2 (9.72-10.00 min); Fraction 3 (10.02-10.56 min).

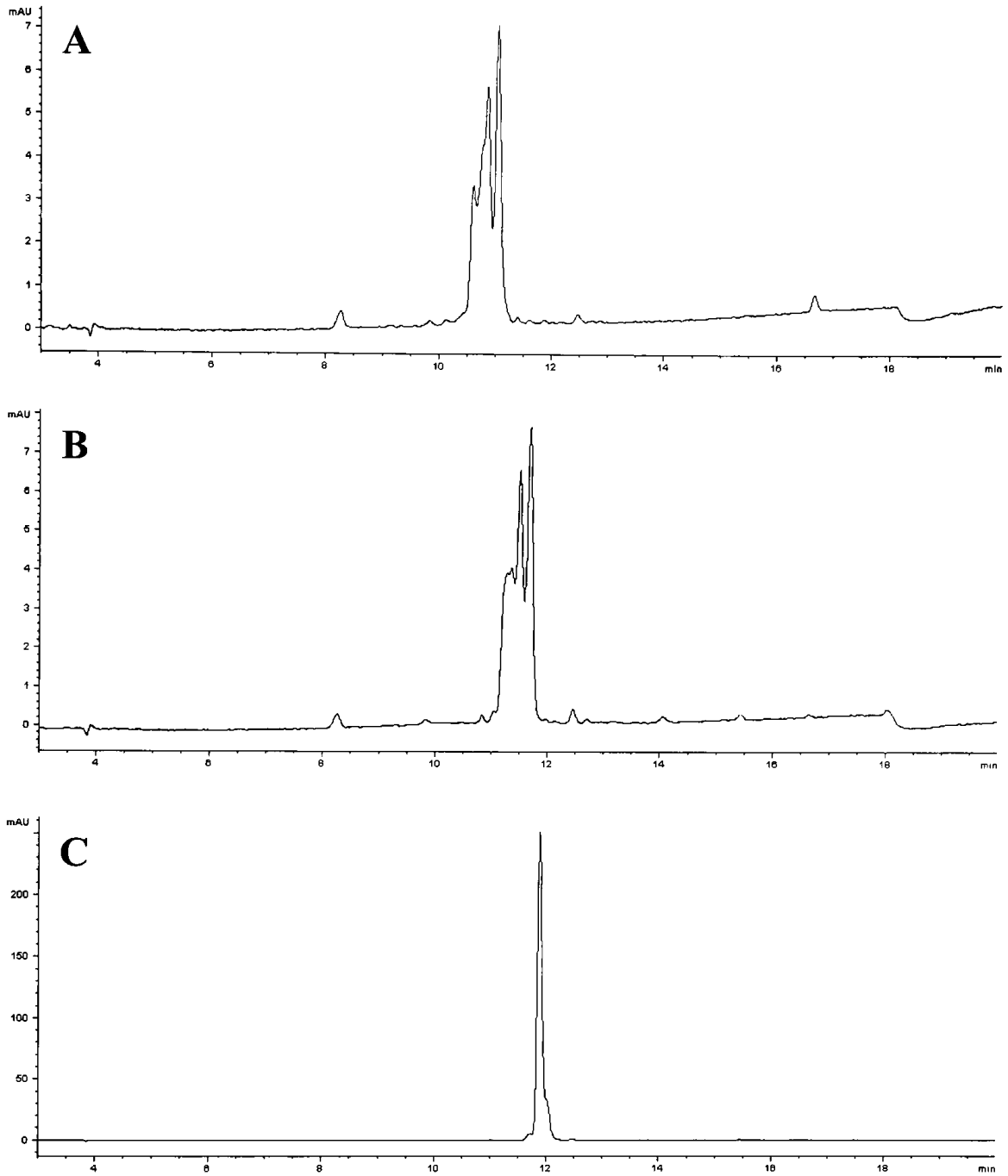


Figure A.5. HPLC Chromatograms of Fractions 4, 5, and 6. Retention times were as follows: Fraction 4 (10.59-11.13 min); Fraction 5 (11.16-11.69 min); Fraction 6 (11.72-12.10 min).

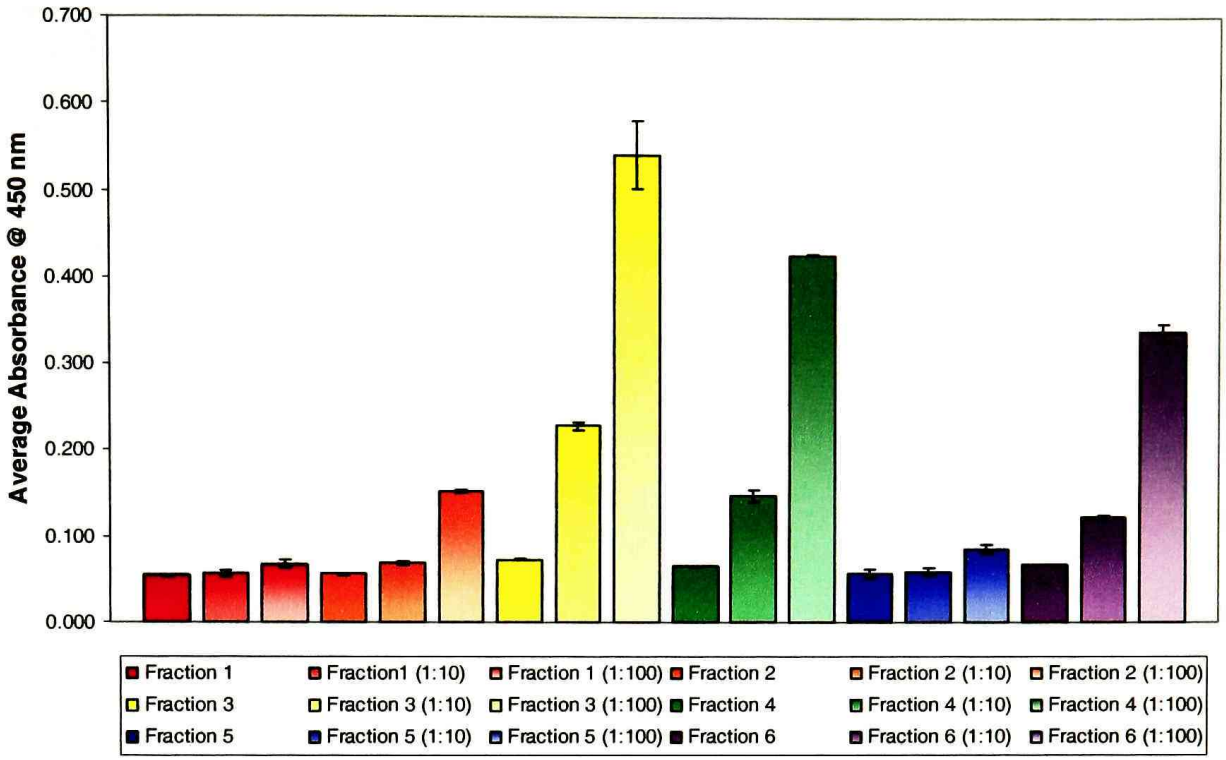


Figure A.6. Macrocyclic Trichothecene-Specific ELISA Results of Six Separated Fractions from *Stachybotrys chartarum* Crude Toxin Extract. Fractions 1 through 6 were normalized and diluted to the concentrations shown above. Average absorbances (± standard deviations) at 450 nm are shown here. Fractions 1, 2, and 5 demonstrated higher ELISA responses than Fractions 3, 4, and 6, but individual trichothecenes could not be isolated as each fraction contained numerous compounds. These six fractions were further fractionated for specific characterization (see below).

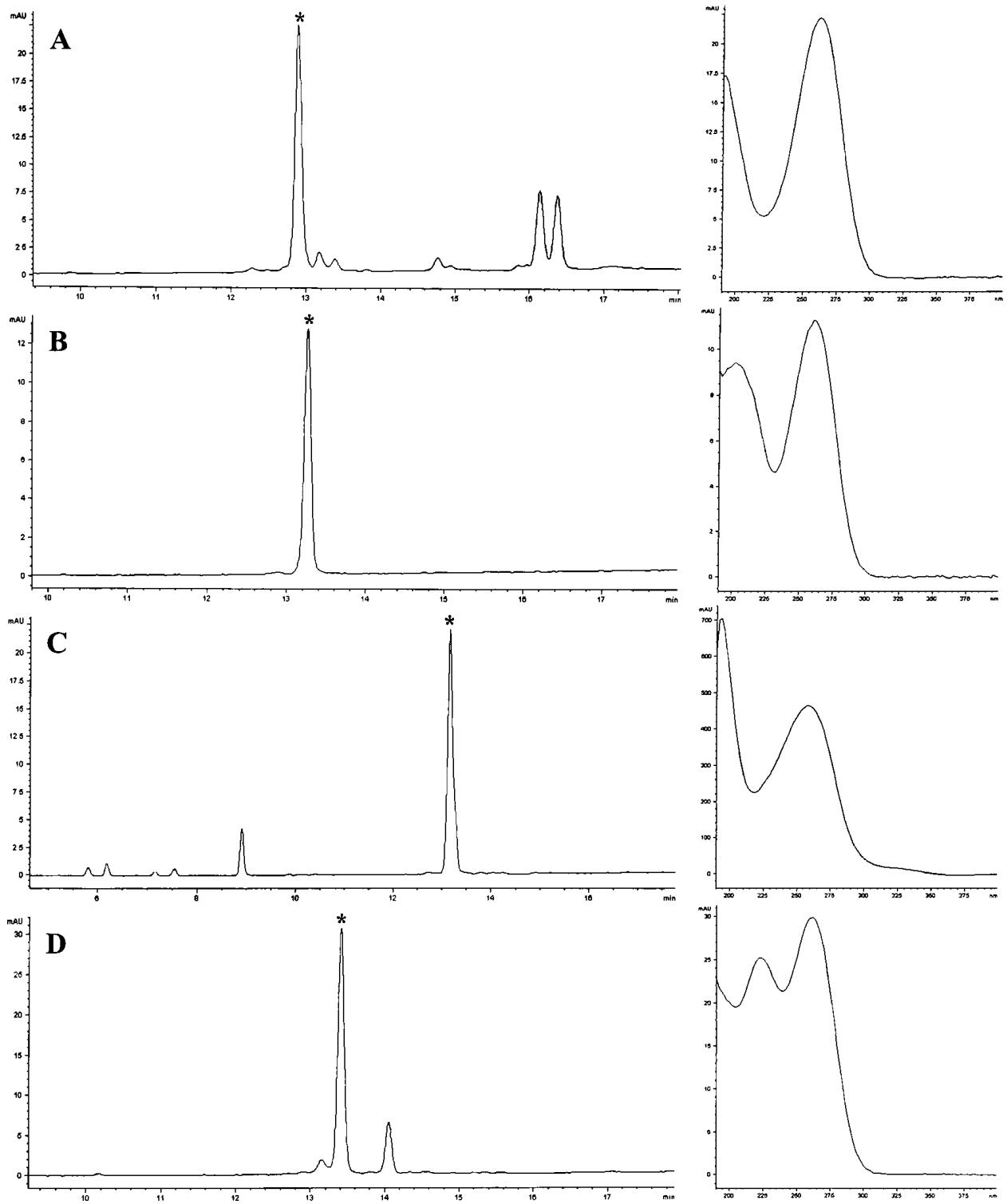


Figure A.7. HPLC Chromatograms and UV Spectra of RP-Separated Compounds from Fraction 1. Four major compounds were separated from Fraction 1 (A-D, in order). Complete separation was not possible as demonstrated by the presence of minor and/or overlapping peaks in each fraction. UV spectrum analysis (200-375 nm) for the major separated compounds (indicated with an *) are shown to the right of each chromatogram. The third fraction here (Panel C) was verified as satratoxin G.

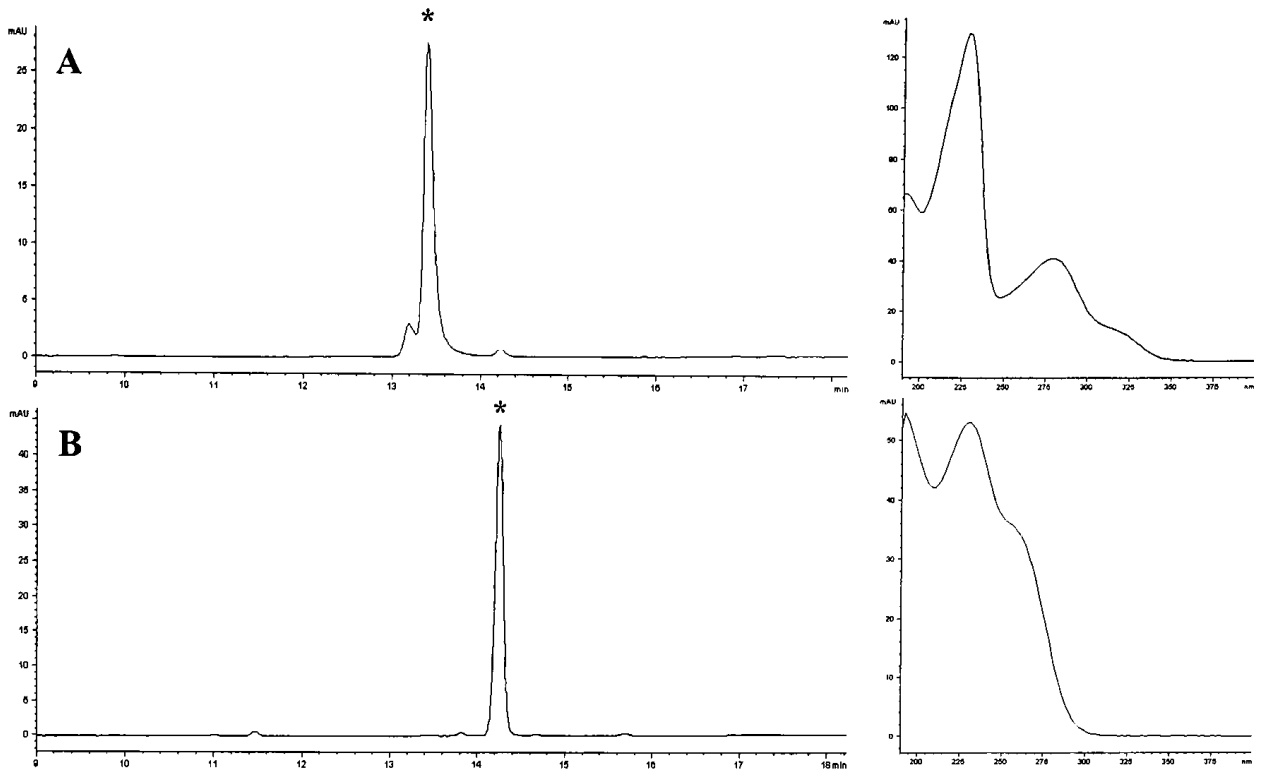


Figure A.8. HPLC Chromatograms and UV Spectra of RP-Separated Compounds from Fraction 2. Two major compounds were separated from Fraction 2 (A-B, in order). Complete separation was not possible as demonstrated by the presence of minor and/or overlapping peaks in each fraction. UV spectrum analysis (200-375 nm) for the major separated compounds (indicated with an *) are shown to the right of each chromatogram. The second fraction here (Panel B) was verified as satratoxin H.

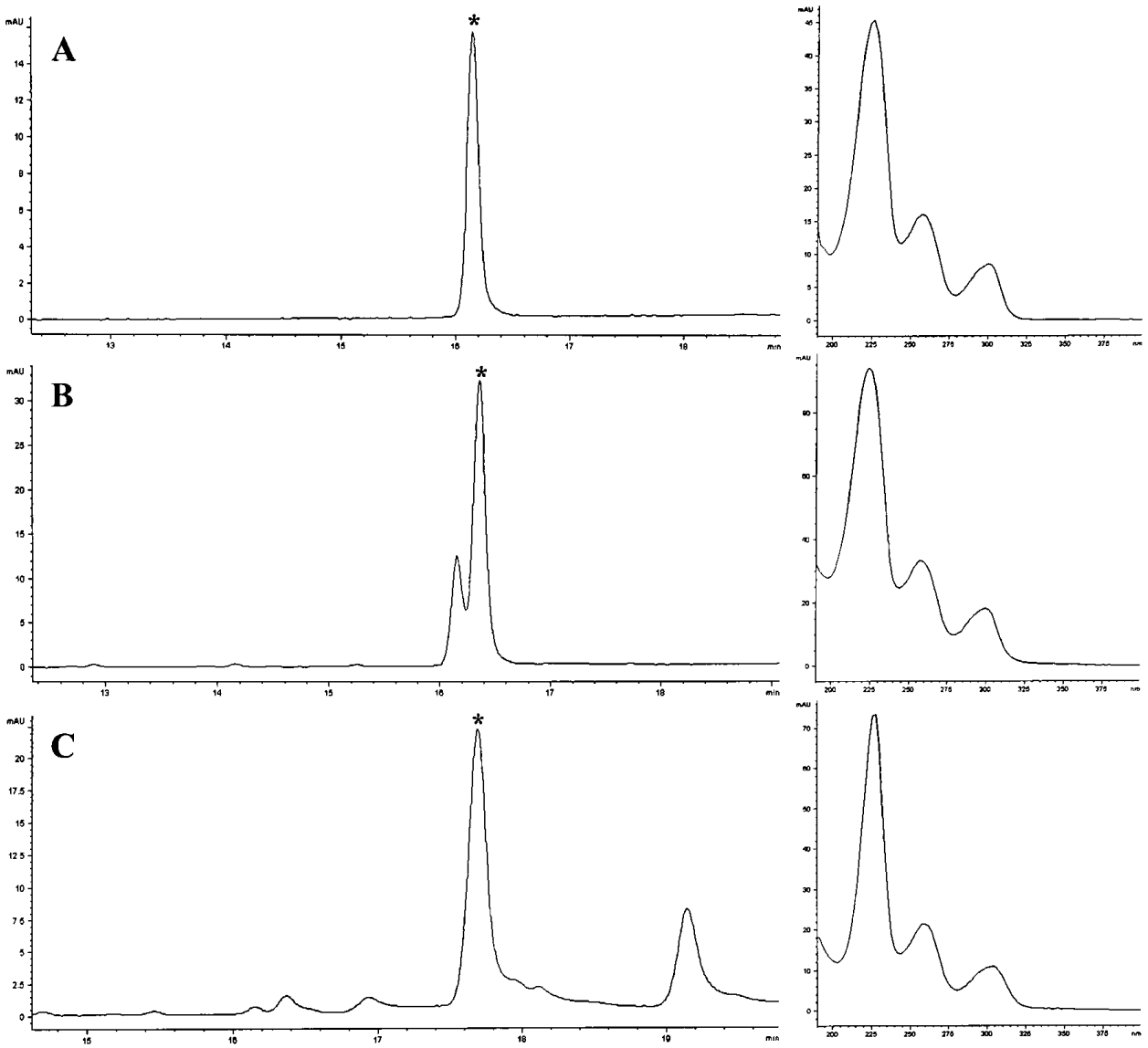


Figure A.9. HPLC Chromatograms and UV Spectra of RP-Separated Compounds from Fraction 3. Three major compounds were separated from Fraction 3 (A-C, in order). Complete separation was not possible as demonstrated by the presence of minor and/or overlapping peaks in each fraction. UV spectrum analysis (200-375 nm) for the major separated compounds (indicated with an *) are shown to the right of each chromatogram.

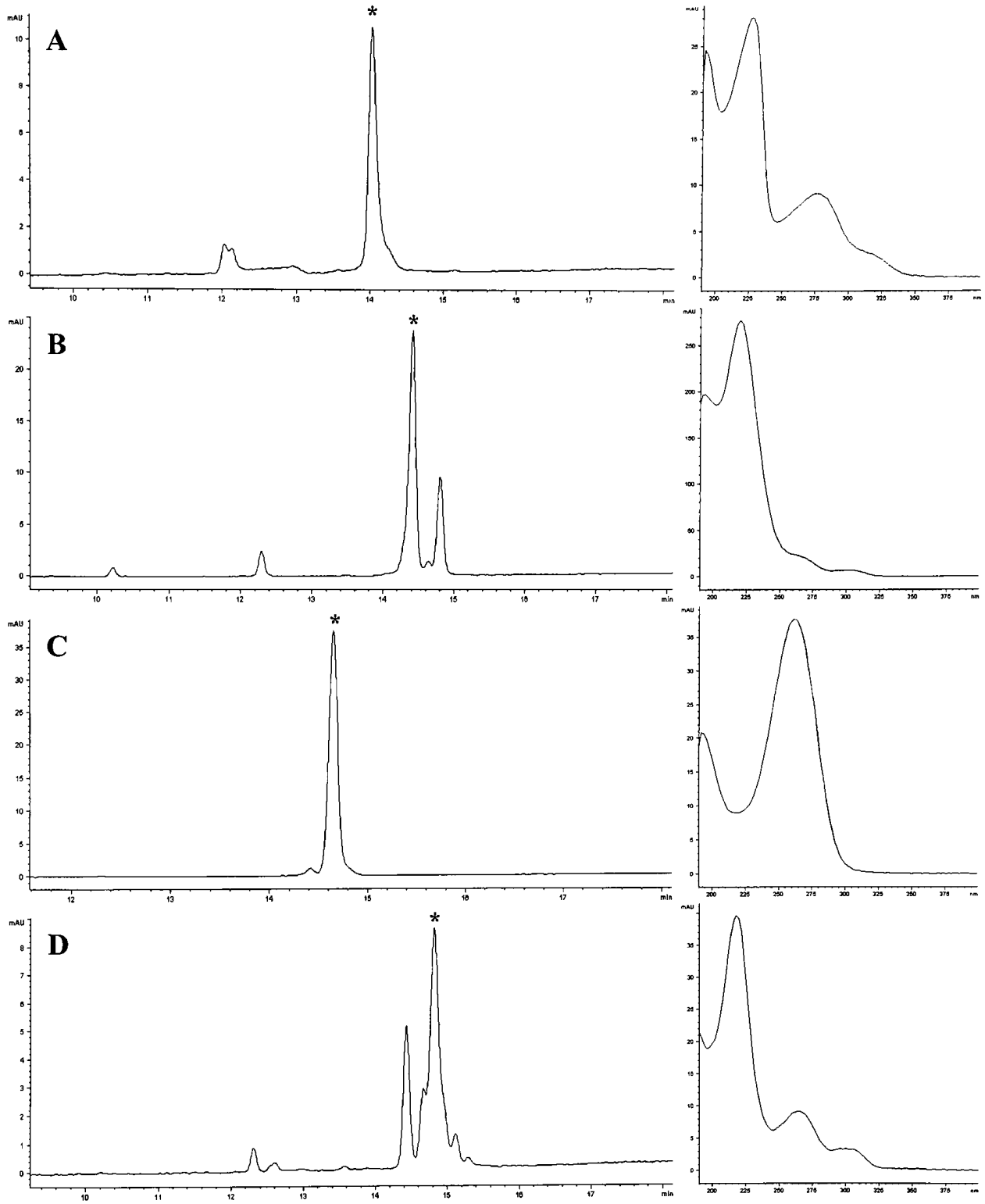


Figure A.10. HPLC Chromatograms and UV Spectra of RP-Separated Compounds from Fraction 4. Eleven major compounds were separated from Fraction 4 (A-K, in order). Complete separation was not possible as demonstrated by the presence of minor and/or overlapping peaks in each fraction. UV spectrum analysis (200-375 nm) for the major separated compounds (indicated with an *) are shown to the right of each chromatogram.

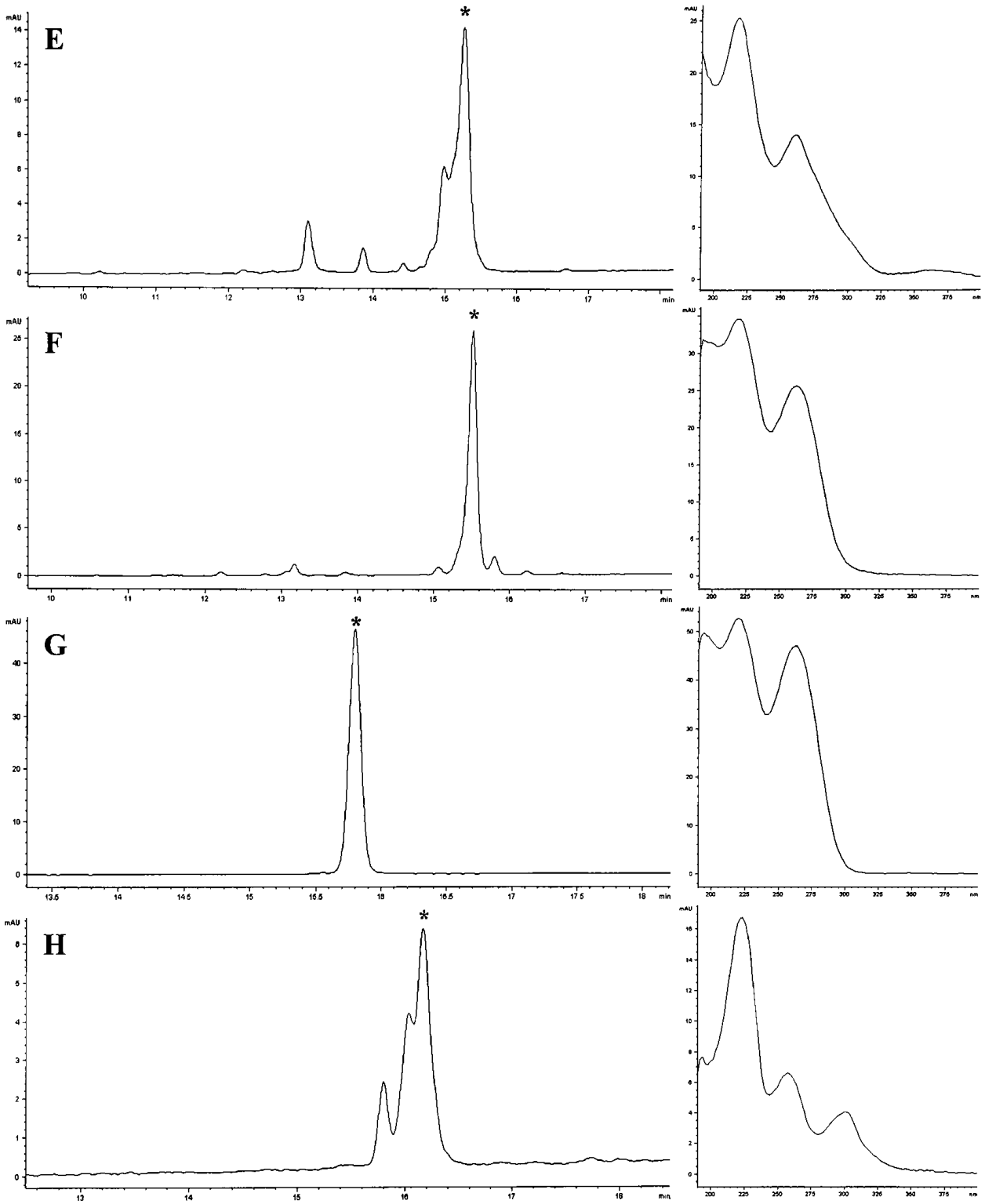


Figure A.10. Continued.

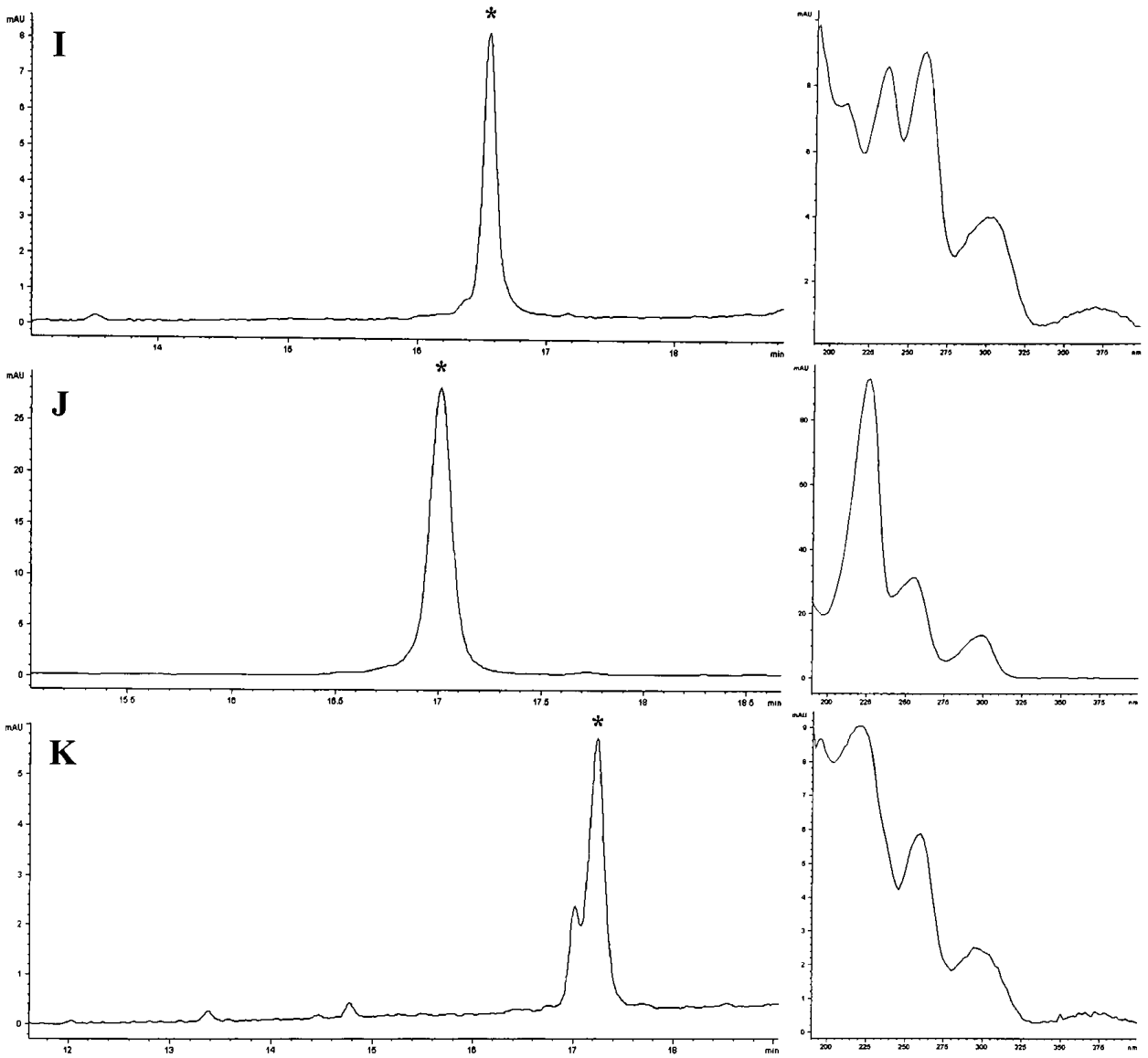


Figure A.10. Continued.

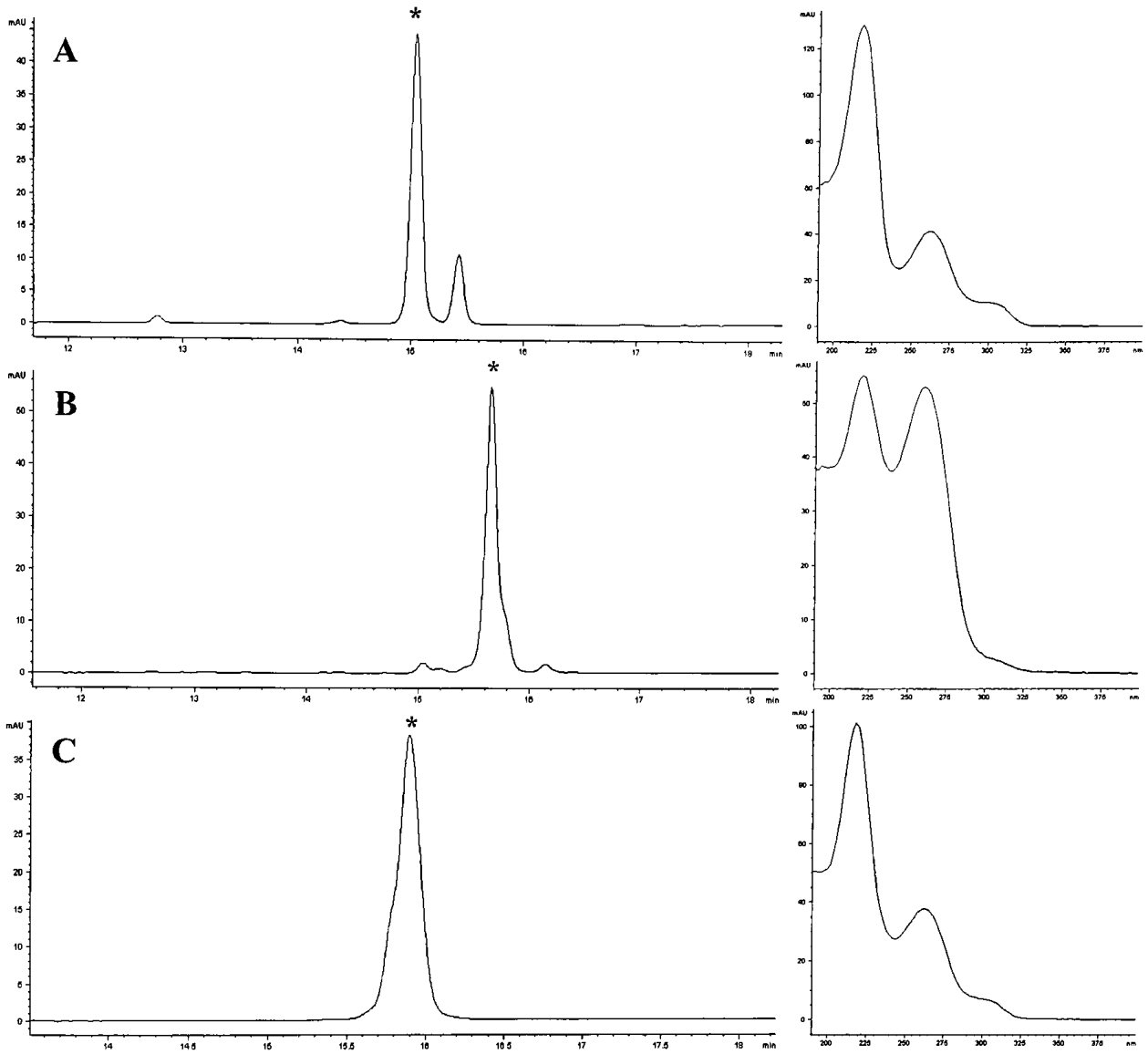


Figure A.11. HPLC Chromatograms and UV Spectra of RP-Separated Compounds from Fraction 5. Six major compounds were separated from Fraction 5 (A-F, in order). Complete separation was not possible as demonstrated by the presence of minor and/or overlapping peaks in each fraction. UV spectrum analysis (200-375 nm) for the major separated compounds (indicated with an *) are shown to the right of each chromatogram.

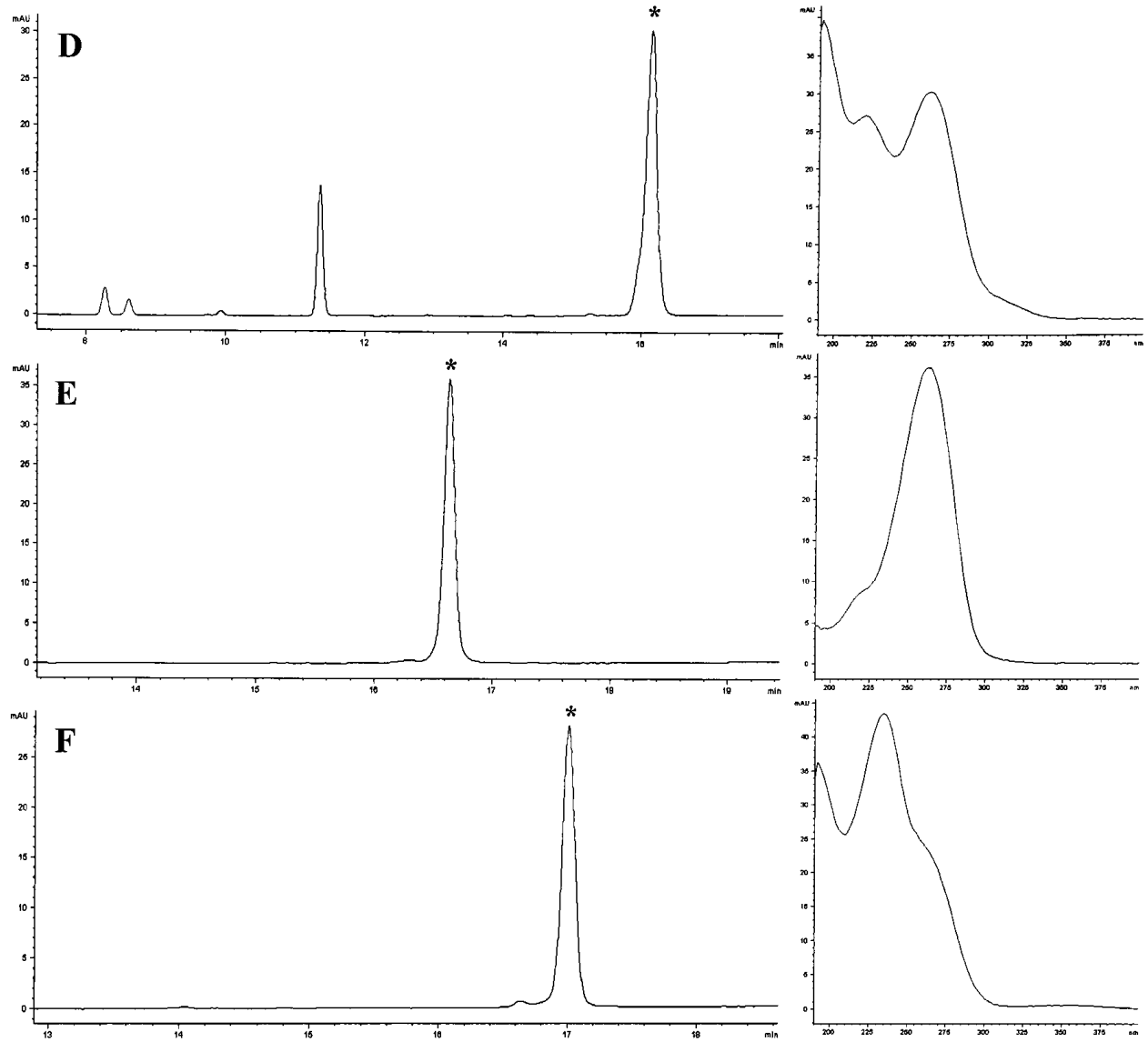


Figure A.11. Continued.

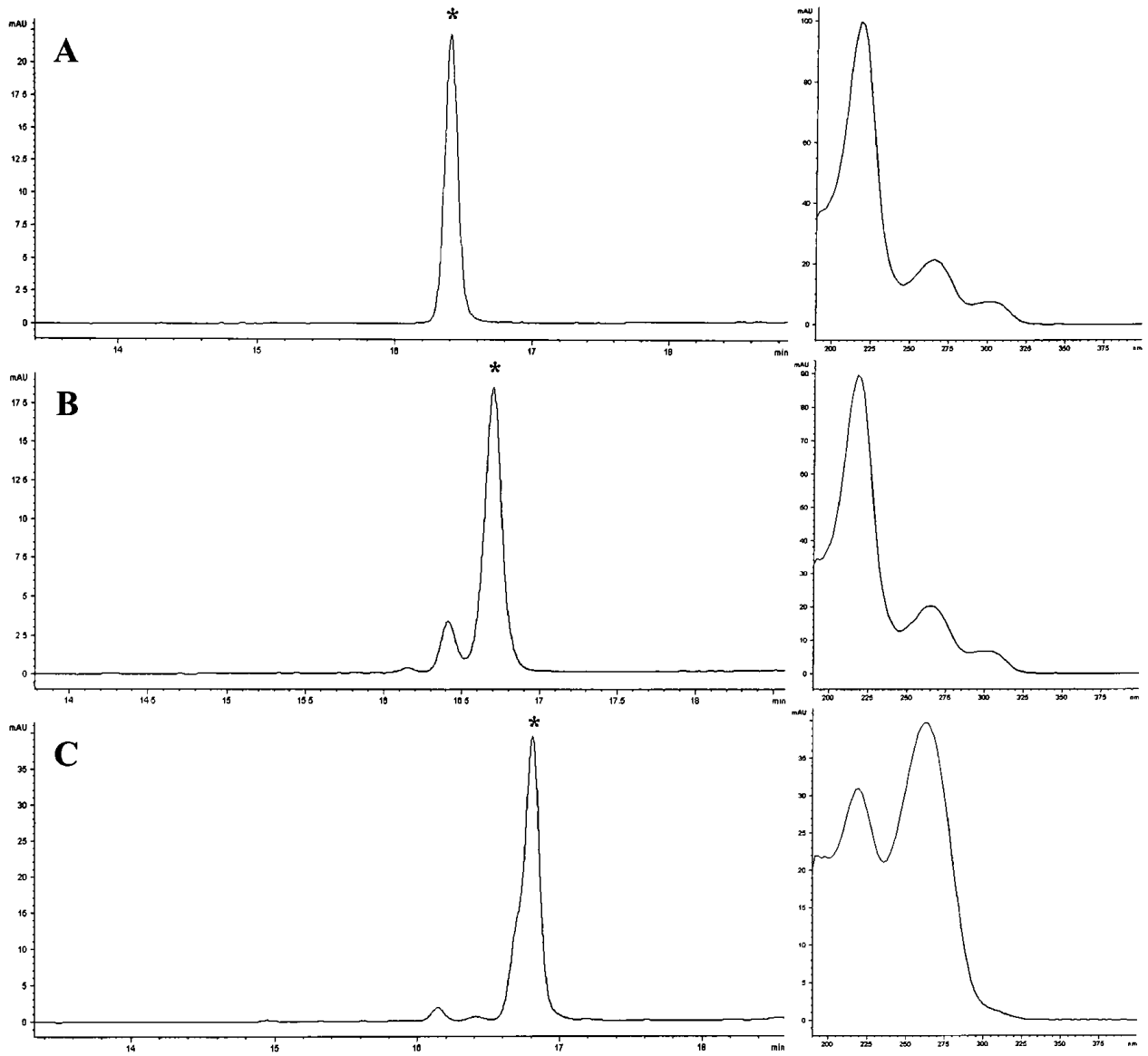


Figure A.12. HPLC Chromatograms and UV Spectra of RP-Separated Compounds from Fraction 6. Three major compounds were separated from Fraction 6 (A-F, in order). Complete separation was not possible as demonstrated by the presence of minor and/or overlapping peaks in each fraction. UV spectrum analysis (200-375 nm) for the major separated compounds (indicated with an *) are shown to the right of each chromatogram.

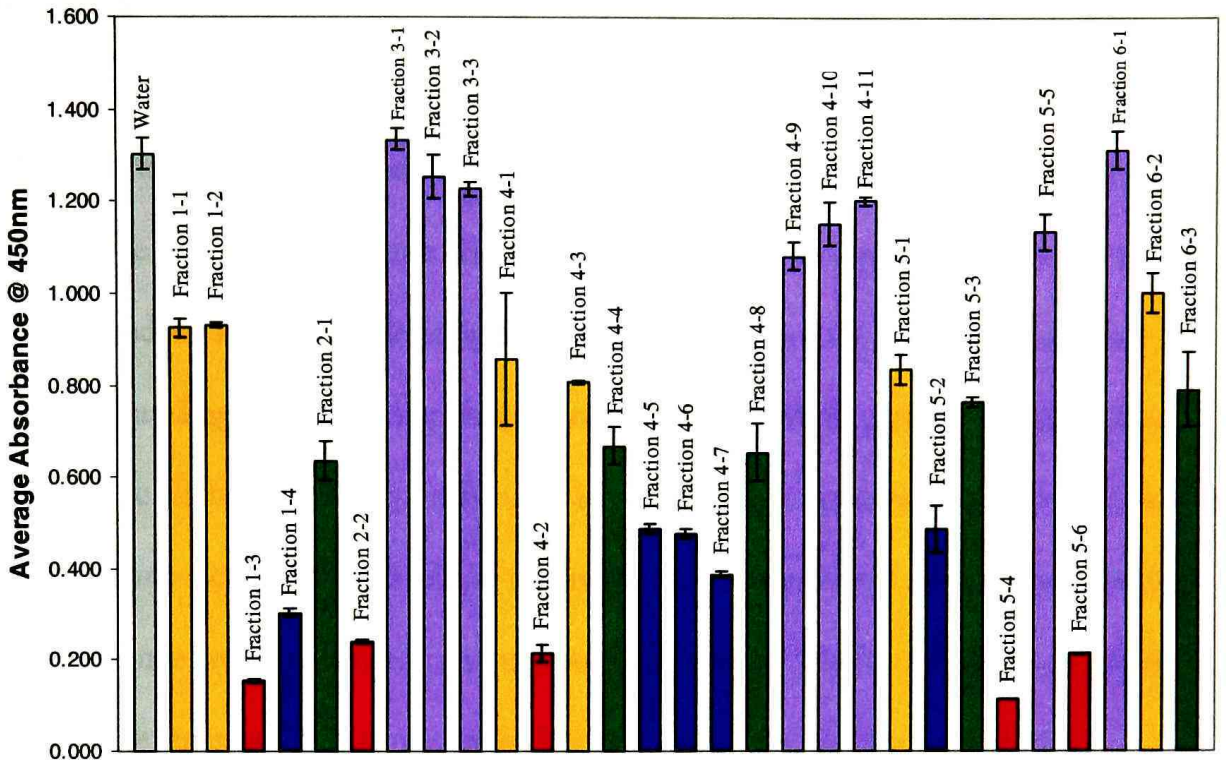


Figure A.13. Macrocyclic Trichothecene-Specific ELISA Results of 29 Separated Fractions from *Stachybotrys chartarum* Crude Toxin Extract. Fractions 1-6 were normalized and diluted in water to approximately 0.3 mAU each. Average absorbances (± standard deviations) at 450 nm are shown here. Fractions are color coordinated based on percent inhibitions versus water alone: 0-20% (purple), 20-40% (yellow), 40-60% (green), 60-80% (blue) and greater than 80% (red). Fractions 1-3 (satratoxin G), 2-2 (satratoxin H), 4-2, 5-4, and 5-6 demonstrated inhibitions greater than 80% and were concluded to be major macrocyclic trichothecenes produced by *S. chartarum*.

Discussion

Using HPLC analysis, we were able to separate and collect various compounds, notably macrocyclic trichothecene mycotoxins, from *Stachybotrys chartarum*. The significance of this is that future characterization of these compounds can be performed to better understand the relationship between the presence of *S. chartarum* in water-damaged buildings and resulting human health effects. We hypothesize that the five macrocyclic trichothecene mycotoxins isolated using our methods play a significant role in *Stachybotrys*-contaminated indoor environments. Separated fractions were relatively pure, but some were composed of numerous compounds. These additional compounds may have influenced ELISA reactivity, but this will not be known until further investigation.

APPENDIX B

ADDITIONAL CONTROLLED AIR SAMPLING SETUP: USE OF *Stachybotrys*-CONTAMINATED RICE FOR THE COLLECTION OF AIRBORNE TRICHOTHECENE MYCOTOXINS ON PARTICULATES SMALLER THAN CONIDIA

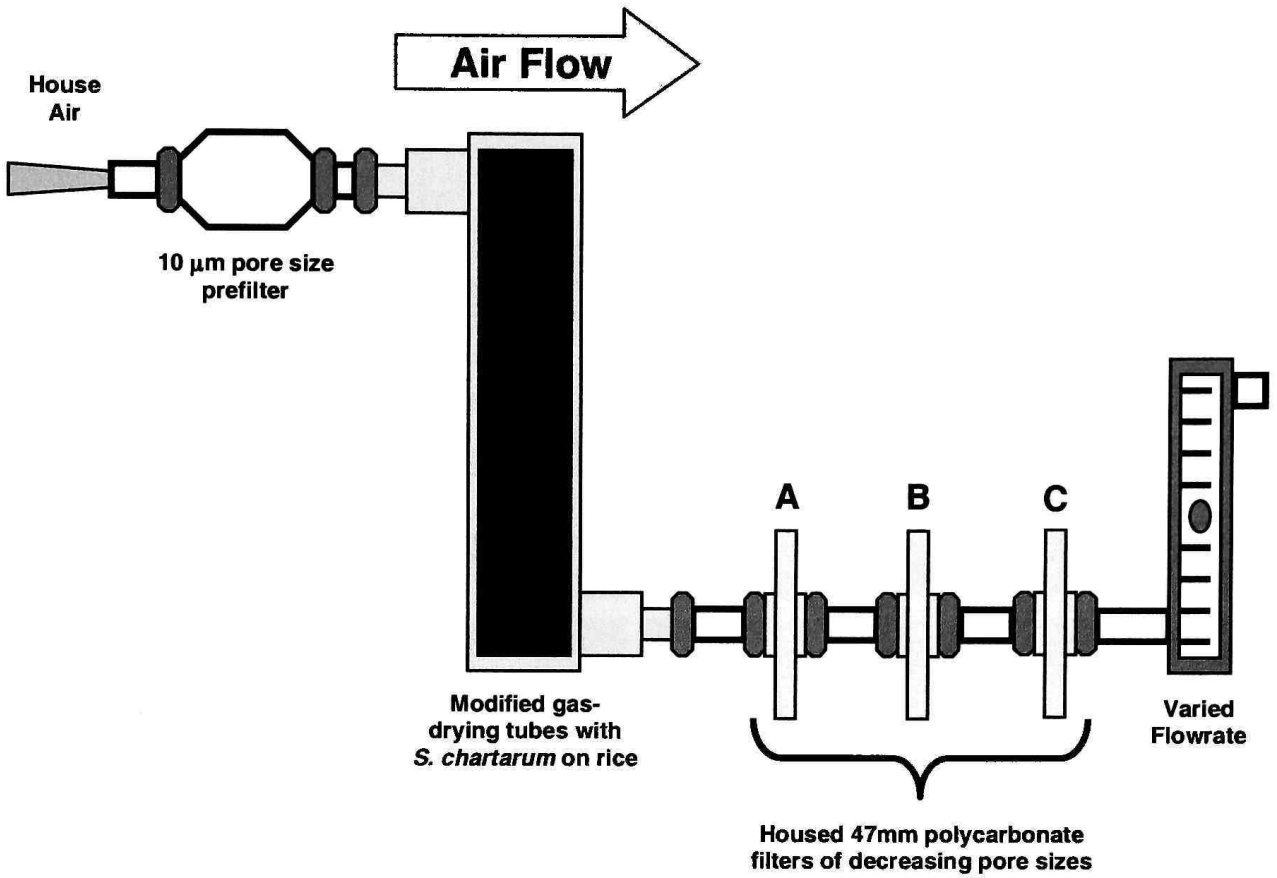


Figure B.1. Alternative Experimental Air Sampling Apparatus. Filtered house air at 10, 20, 30, or 45 LPM was passed through rice with confluent *Stachybotrys chartarum*-growth for different periods of time (outlined in Table B.1). This was done using one gas-drying tube full of the contaminated rice. Particles were separated and collected on 47 mm diameter polycarbonate membrane filters with pore sizes of (A) 20.0 µm, (B) 5.0 µm, and (C) 0.4 µm and later analyzed for the presence of macrocyclic trichothecenes as described in the text (Chapter II).

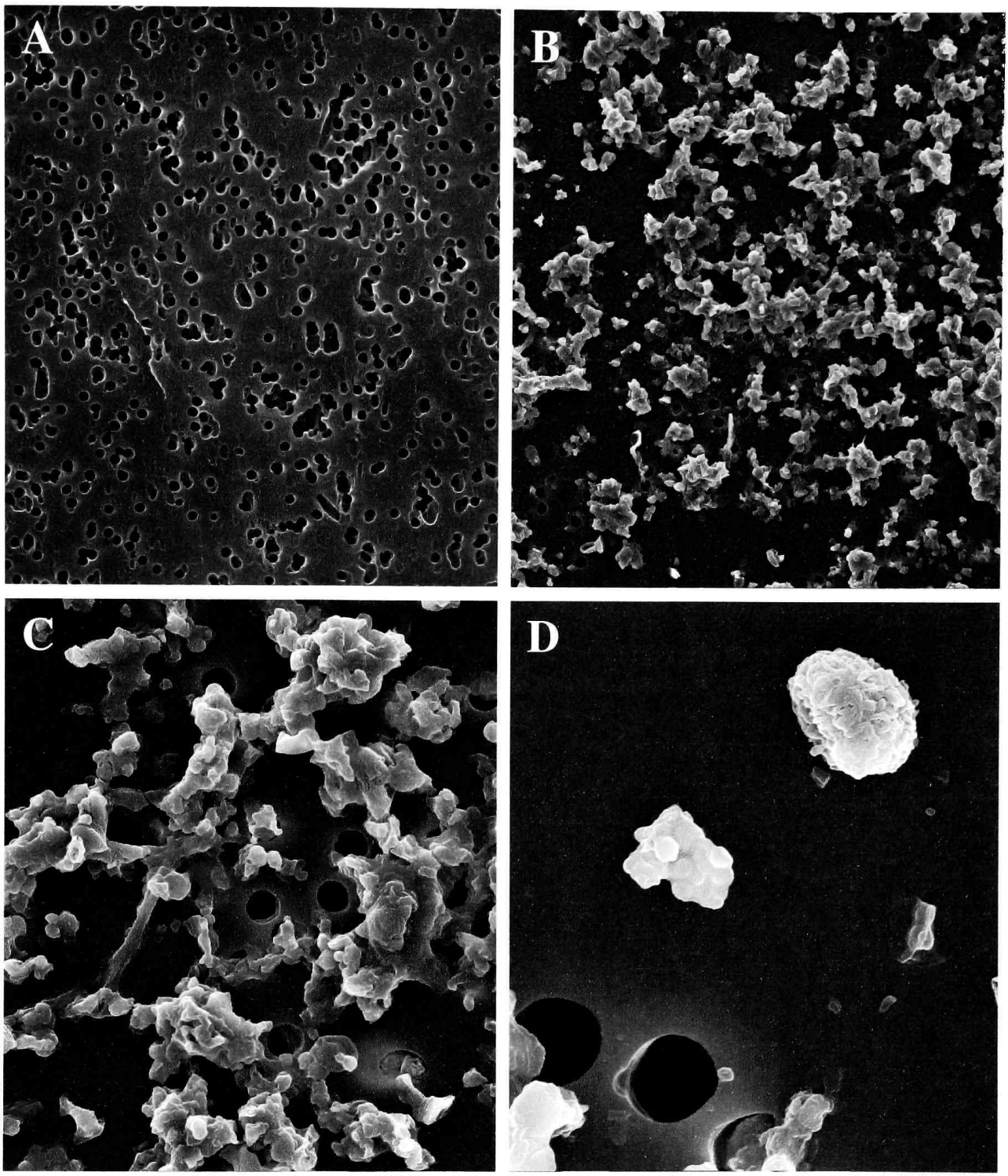


Figure B.2. Select Scanning Electron Micrographs of 5.0 μm Pore Size Polycarbonate Membrane Filters Used in the Alternative Air Sampling Setup. A marked difference was seen between a sterile unused filter and one after a one hour sampling (A and B, respectively; 100x magnification). These experiments were successful in capturing various types of particulate matter (C; 300x magnification) including desiccated conidia (D, upper right; 800x magnification). Filters with a 20 μm pore size were overloaded with particulate matter and are not shown here. [Electron microscopy performed by Dr. Dennis Kunkel, Dennis Kunkel Microscopy, Inc.]

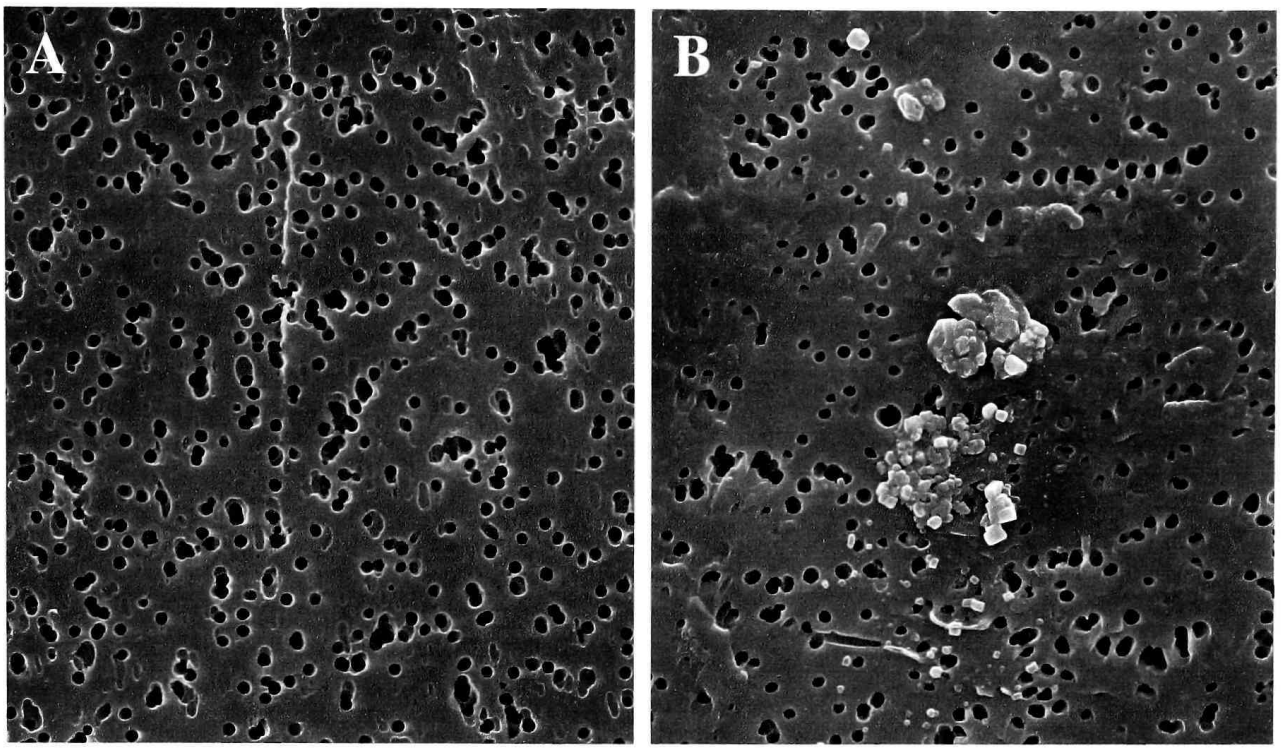


Figure B.3. Select Scanning Electron Micrographs of 0.4 μm Pore Size Polycarbonate Membrane Filters Used in the Alternative Air Sampling Setup. Collected particulate matter was in low abundance on the final filters in the air sampling setup. No intact conidia were captured, but highly respirable particulate matter was identified on the experimental filters (B; 1000x magnification). Image A represents a sterile, unused filter (1000x magnification).

Table B.1. Competitive ELISA results of polycarbonate filter extracts – *Stachybotrys*-contaminated rice setup.

Filter pore size[a]	Sampling Time and Flowrate[b]	Average % Inhibition[c]	Average Trichothecene Equivalent[d]
20.0		95.91 ± 0.11*	>500
5.0	1 hour at 10 LPM	47.09 ± 2.87*	0.75 ± 0.11
0.4		10.81 ± 3.74*	0.19 ± 0.02
20.0		95.88 ± 0.38*	>500
5.0	1 hour at 20 LPM (Trial 1)	64.46 ± 0.70*	2.12 ± 0.11
0.4		36.50 ± 3.94*	0.47 ± 0.08
20.0		96.32 ± 0.24*	>500
5.0	1 hour at 20 LPM (Trial 2)	24.50 ± 2.33*	0.29 ± 0.02
0.4		29.33 ± 0.63*	0.35 ± 0.01
20.0		95.65 ± 0.58*	>500
5.0	3 hours at 20 LPM	54.26 ± 8.10*	1.19 ± 0.45
0.4		21.12 ± 9.24*	0.28 ± 0.09
20.0		96.36 ± 0.07*	>500
5.0	1 hour at 30 LPM (Trial 1)	24.68 ± 1.17*	0.29 ± 0.01
0.4		67.19 ± 2.17*	2.69 ± 0.50
20.0		96.20 ± 0.17*	>500
5.0	1 hour at 30 LPM (Trial 2)	21.62 ± 2.16*	0.27 ± 0.02
0.4		36.52 ± 0.51*	0.47 ± 0.01

Table B.1. Continued.

Filter pore size[a]	Sampling Time and Flowrate[b]	Average % Inhibition[c]	Average Trichothecene Equivalent[d]
20.0		96.29 ± 0.00*	>500
5.0	1 hour at 45 LPM	27.53 ± 8.75*	0.34 ± 0.12
0.4		32.04 ± 1.88*	0.39 ± 0.03

[a]In μm. Filters are grouped based on order of the series and sampling time.

[b]LPM; liters per minute. Certain trials were run twice (indicated in parentheses).

[c]Results are based on extracts of sterile, unexposed membrane filters (of the respective pore size) in 5% methanol in PBS. Values represent triplicate wells. Standard deviations are shown. Those values significant as determined by a one-way ANOVA ($P<0.05$) are noted with an *.

[d]In ng/ml. Values are semi-quantitative and are based on the macrocyclic trichothecene standard curve presented in Figure 2.3. Statistical analyses were performed as described for percent inhibition comparisons. Values represent triplicate wells.

APPENDIX C
SUPPLEMENTARY BUILDING DATA:
AIRBORNE CONIDIA COUNTS
AND IMAGES

APPENDIX C

Table C.1. Airborne conidia types and counts isolated from Building 1* for each sampling time and condition.

Room[a]	Sampling Time and Conditions[b]	Airborne Fungal Genera - Conidia Counts per m^3 of Air[c]										Debris Count[d]
		Alt	Asco	*Bip*	*Clado*	*Curv*	*Fus*	*Memn*	*Nigro*	*Pen/Asp*	*Stachy*	
OSA	NA	774	1065	0	2548	0	0	0	65	12226	0	Medium
Kitchen	120 min - Static	32	5	0	1645	0	0	0	0	11226	0	Very Light
	30 min - Disturbed	3710	32	0	161	0	0	0	0	3710	0	Very Light
	10 min - Disturbed	97	581	0	1355	0	161	0	0	10677	452	Medium
TV Room	120 min - Static	548	1484	0	1548	65	0	0	0	48613	1226	Heavy
	120 min - Disturbed	323	4065	0	1000	0	0	0	65	17129	5032	Medium
	30 min - Disturbed	0	0	0	97	0	32	0	32	3935	0	Very Light
	10 min - Disturbed	161	2452	0	710	0	226	0	0	28290	2710	Medium
Bedroom	120 min - Static	258	1161	0	161	0	0	0	0	9419	0	Medium
	120 min - Disturbed	226	3355	0	1323	0	0	0	32	3968	0	Medium
	30 min - Disturbed	258	806	0	1065	0	0	0	0	6065	0	Light
	10 min - Disturbed	355	5677	0	2355	97	0	0	0	7935	0	Medium

*This building was referred to in Table 3.2 as Test 1

[a]OSA (Outside air) was considered normal and served as a control for sampling.

[b]Rooms were sampled under static and/or disturbed conditions for the noted times. Air disturbance was accomplished using 20-inch box fans on a "high" setting. Disturbance was allowed for 5 minutes prior to starting the SpinCon collection. NA; not applicable.

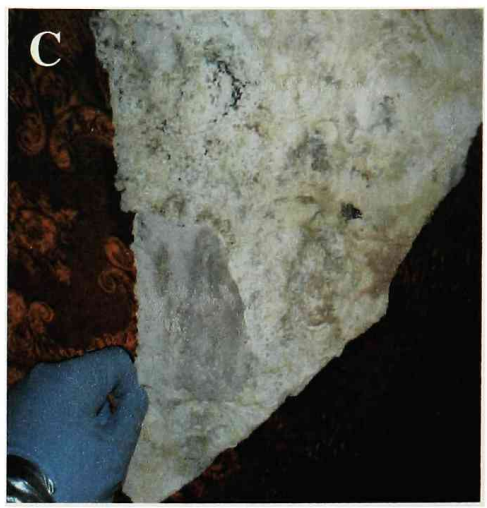
[c]5-minute Allergenco spore traps were taken to assess airborne fungal conidia types and concentrations as well as to qualitate the amount of debris present. For static conditions, samples were taken just prior to SpinCon collection. For disturbed conditions, Allergencos were started 10 minutes prior to the end of the sampling period. Key: *Alt - Alternaria*; Asco - Ascospores (most likely *Chaetomium*, but unable to confirm); *Bip - Bipolaris*; *Clado - Cladosporium*; *Curv - Curvularia*; *Fus - Fusarium*; *Memn - Memnoniella*; *Nigro - Nigrospora*; *Pen/Asp - Penicillium/Aspergillus*-like (unable to confirm); *Stachy – Stachybotrys*

[d]Debris was defined as non-identifiable particles and were qualitated based on the approximate percentage of the viewed field covered by such particles: Very Light (<20%), Light (21-40%), Medium (41-60%), Heavy (61-80%), and Very Heavy (>80%).

Figure C.1. Select Images from Building 1. Heavy water damage led to severe mold growth throughout Building 1. *Stachybotrys* contamination was heaviest in the kitchen behind appliances (A) and under wallpapering in the entertainment room (C). Large areas of drywall and wood flooring were also found to be contaminated (B and D, respectively). Air sampling was performed under static and disturbed conditions using a high volume wet concentrator (E, F). Air disturbance was accomplished using box fans placed in the corners of each sampled room (E).


A


B


C


D


E


F

Table C.2. Airborne conidia types and counts isolated from Building 2 for each sampling time and condition.

Room[a]	Sampling Time and Conditions[b]	Airborne Fungal Genera - Conidia Counts per m^3 of Air[c]										Debris Count[d]
		Alt	*Asco*	*Bip*	*Clado*	*Curv*	*Fus*	*Memn*	*Nigro*	*Pen/Asp*	*Stachy*	
OSA	NA	129	258	0	355	194	0	0	0	3161	0	Medium
Main Entry	120 min - Static	226	1548	0	1000	0	0	0	32	5806	1161	Heavy
	120 min - Disturbed											THTC
	30 min - Disturbed	129	613	0	1387	0	0	0	65	6581	323	Medium
	10 min - Disturbed	65	710	0	1065	0	0	0	0	5839	226	Heavy
Kitchen	120 min - Static	226	774	0	194	32	0	0	0	2387	3097	Heavy
	120 min - Disturbed						-					THTC
	30 min - Disturbed	-										THTC

[a]OSA (Outside air) was considered normal and served as a control for sampling.

[b]Rooms were sampled under static and/or disturbed conditions for the noted times. Air disturbance was accomplished using 20-inch box fans on a "high" setting. Disturbance was allowed for 5 minutes prior to starting the SpinCon collection. NA; not applicable.

[c]5-minute Allergenco spore traps were taken to assess airborne fungal conidia types and concentrations as well as to qualitate the amount of debris present. For static conditions, samples were taken just prior to SpinCon collection. For disturbed conditions, Allergencos were started 10 minutes prior to the end of the sampling period. Key: *Alt - Alternaria*; Asco - Ascospores (most likely *Chaetomium*, but unable to confirm); *Bip - Bipolaris*; *Clado - Cladosporium*; *Curv - Curvularia*; *Fus - Fusarium*; *Memn - Memnoniella*; *Nigro - Nigrospora*; *Pen/Asp - Penicillium/Aspergillus*-like (unable to confirm); *Stachy – Stachybotrys.*

[d]Debris was defined as non-identifiable particles and were qualitated based on the approximate percentage of the viewed field covered by such particles: Very Light (<20%), Light (21-40%), Medium (41-60%), Heavy (61-80%), and Very Heavy (>80%). THTC; Too heavy to count (unable to identify and enumerate conidia).

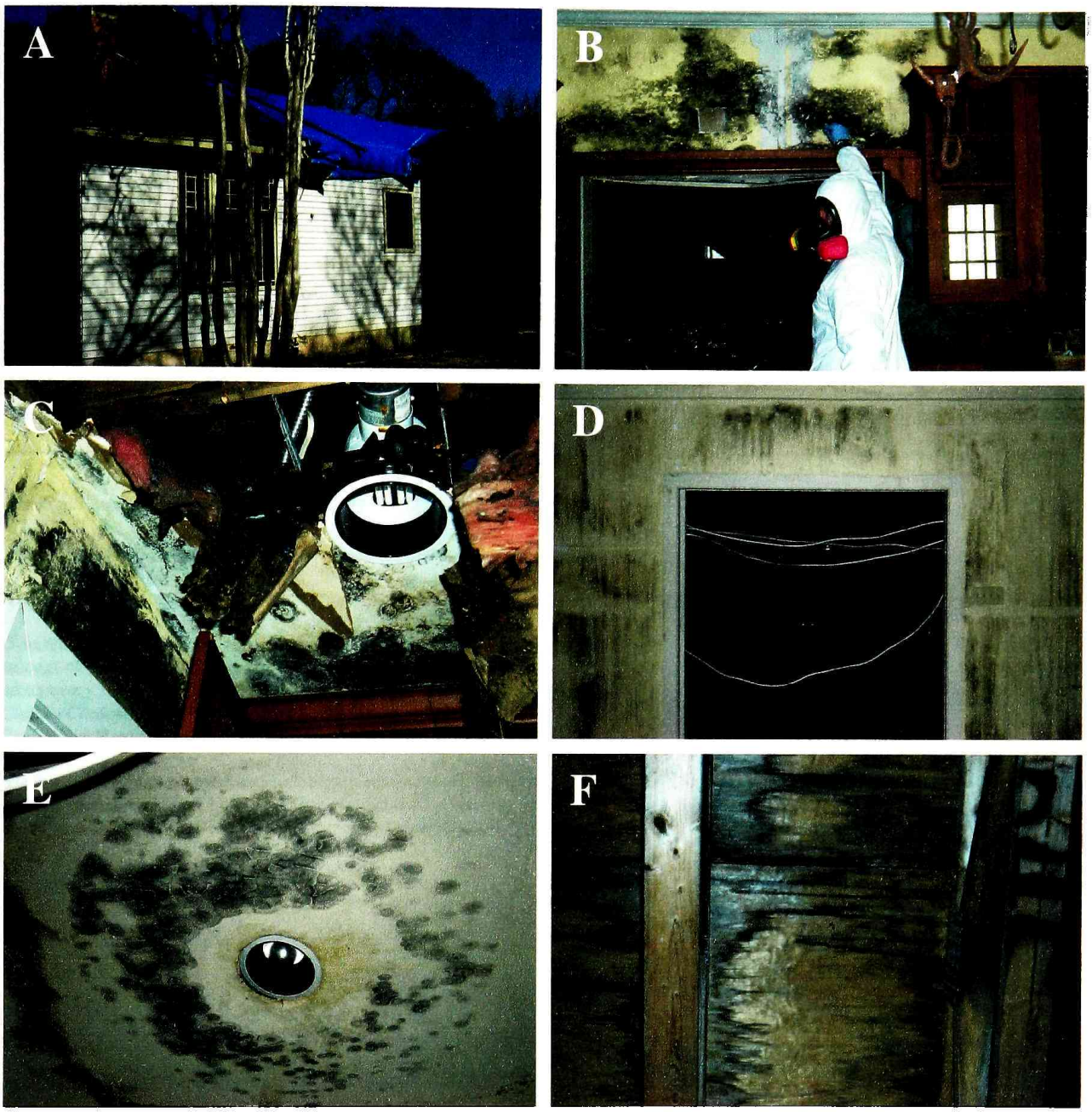


Figure C.2. Select Images from Building 2. Heavy *Stachybotrys* growth was noted on most visible surfaces throughout this building (B-F). Sampled areas included the kitchen (B and C) and the main entry room (D). Fungal growth was a result of extreme condensation buildup and other instances of water damage due to incomplete roof renovation (A).

Table C.3. Airborne conidia types and counts isolated from Building 3* for each sampling time and condition.

Room[a]	Sampling Time and Conditions[b]	Airborne Fungal Genera - Conidia Counts per m^3 of Air[c]										D C
		Alt	*Asco*	*Bip*	*Clado*	*Curv*	*Fus*	*Memn*	*Nigro*	*Pen/Asp*	*Stachy*	
OSA	NA	677	871	0	3129	0	0	0	32	3226	0	I
Laundry Room	120 min - Static	194	129	0	65	0	0	0	0	4032	8839	M
	10 min - Agg 1					-					16968	V.
	10 min - Agg 2	-									14355	V.
	10 min - Agg 3	-				-	-					T

*This building was referred to in Table 3.2 as Test 2

[a]OSA (Outside air) was considered normal and served as a control for sampling.

[b]This room was sampled under static conditions or during aggressive sampling (Agg) for the noted times. NA; not applicable.

[c]5-minute Allergenco spore traps were taken to assess airborne fungal conidia types and concentrations as well as to qualitate the amount of debris present. For static conditions, samples were taken just prior to SpinCon collection. During remediation, Allergencos taken every 10 minutes. Key: *Alt - Alternaria*; Asco - Ascospores (most likely *Chaetomium*, but unable to confirm); *Bip - Bipolaris*; *Clado - Cladosporium*; *Curv - Curvularia*; *Fus - Fusarium*; *Memn - Memnoniella*; *Nigro - Nigrospora*; *Pen/Asp - Penicillium/Aspergillus*-like (unable to confirm); *Stachy – Stachybotrys.*

[d]Debris was defined as non-identifiable particles and were qualitated based on the approximate percentage of the viewed field covered by such particles: Very Light (<20%), Light (21-40%), Medium (41-60%), Heavy (61-80%), and Very Heavy (>80%). THTC; Too heavy to count (unable to identify and enumerate conidia).

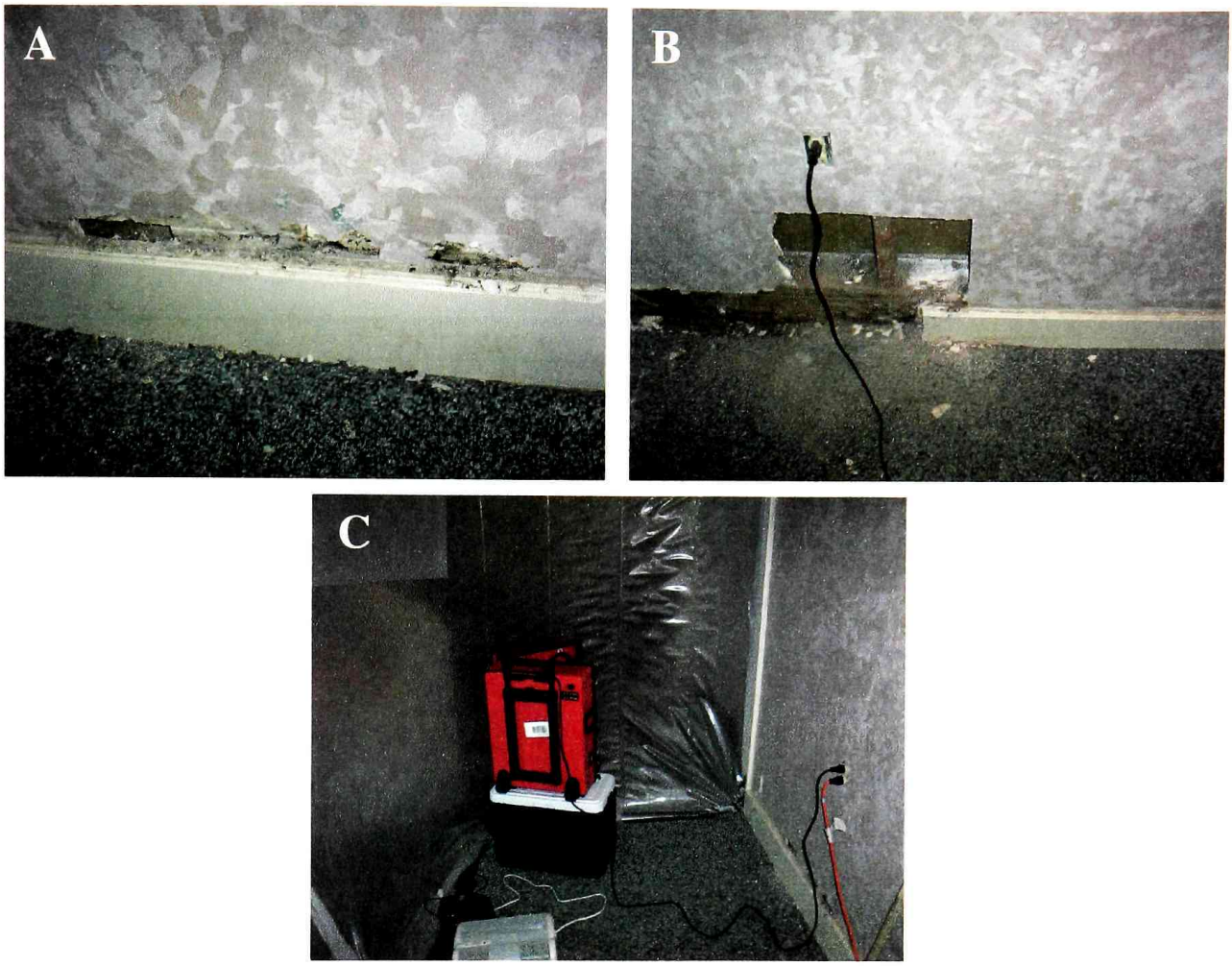


Figure C.3. Select Images from Building 4. Mold (*S. chartarum*) and water damage was minor in this building (A). Most growth was not visible until invasive inspection (B). Air sampling was performed during the invasive inspection that resulted in the production of large amounts of dust and debris (including particulates arising from the *Stachybotrys* growth). To prevent contamination throughout the building, sampling was performed in a contained area under negative pressure (C).

Table C.4. Airborne conidia types and counts isolated from Building 5 for each sampling time and condition.

Room[a]	Sampling Time and Conditions[b]	Airborne Fungal Genera - Conidia Counts per m^3 of Air[c]										Debris Count[d]
		Alt	*Asco*	*Bip*	*Clado*	*Curv*	*Fus*	*Memn*	*Nigro*	*Pen/Asp*	*Stachy*	
OSA	NA	97	129	0	0	0	0	0	0	355	0	Very Light
Front Bathroom	120 min - Static	32	32	0	323	32	0	0	0	1129	0	Light
Back Bathroom	120 min - Static	65	548	0	65	0	0	0	0	355	0	Light

[a]OSA (Outside air) was considered normal and served as a control for sampling.

[b]These rooms were sampled under static conditions for the noted times. NA; not applicable.

[c]5-minute Burkard spore traps were taken to assess airborne fungal conidia types and concentrations as well as to qualitate the amount of debris present. Samples were taken just prior to SpinCon collection. Key: *Alt - Alternaria*; Asco - Ascospores (most likely *Chaetomium*, but unable to confirm); *Bip - Bipolaris*; *Clado - Cladosporium*; *Curv - Curvularia*; *Fus - Fusarium*; *Memn - Memnoniella*; *Nigro - Nigrospora*; *Pen/Asp - Penicillium/Aspergillus*-like (unable to confirm); *Stachy – Stachybotrys*.

[d]Debris was defined as non-identifiable particles and were qualitated based on the approximate percentage of the viewed field covered by such particles: Very Light (<20%), Light (21-40%), Medium (41-60%), Heavy (61-80%), and Very Heavy (>80%).

Figure C.4. Select Images from Building 5. Water damage as a result of numerous leaks and construction problems was heavy throughout this building (A-D). Visible mold growth was minor, but *Stachybotrys* was identified (B).

Table C.5. Airborne conidia types and counts isolated from Building 6 for each sampling time and condition.

Room[a]	Sampling Time and Conditions[b]	Airborne Fungal Genera - Conidia Counts per m^3 of Air[c]										Debris Count[d]
		Alt	*Asco*	*Bip*	*Clado*	*Curv*	*Fus*	*Memn*	*Nigro*	*Pen/Asp*	*Stachy*	
OSA	NA	129	97	0	1710	0	0	0	32	1645	0	Light
Kitchen	120 min - Static	0	32	0	226	0	0	0	0	1581	0	Light
	10 min - Disturbed	0	419	0	129	0	0	0	0	2548	32	Light
Garage	120 min - Static	0	161	0	419	0	0	0	0	4677	0	Medium
	10 min - Disturbed	65	516	0	1065	0	0	0	0	10871	0	Medium

[a]OSA (Outside air) was considered normal and served as a control for sampling.

[b]Rooms were sampled under static and/or disturbed conditions for the noted times. Air disturbance was accomplished using 20-inch box fans on a "high" setting. Disturbance was allowed for 5 minutes prior to starting the SpinCon collection. NA; not applicable.

[c]5-minute Allergenco spore traps were taken to assess airborne fungal conidia types and concentrations as well as to qualitate the amount of debris present. For static conditions, samples were taken just prior to SpinCon collection. For disturbed conditions, Allergencos were started 10 minutes prior to the end of the sampling period. Key: *Alt - Alternaria*; Asco - Ascospores (most likely *Chaetomium*, but unable to confirm); *Bip - Bipolaris*; *Clado - Cladosporium*; *Curv - Curvularia*; *Fus - Fusarium*; *Memn - Memnoniella*; *Nigro - Nigrospora*; *Pen/Asp - Penicillium/Aspergillus*-like (unable to confirm); *Stachy – Stachybotrys.*

[d]Debris was defined as non-identifiable particles and were qualitated based on the approximate percentage of the viewed field covered by such particles: Very Light (<20%), Light (21-40%), Medium (41-60%), Heavy (61-80%), and Very Heavy (>80%).

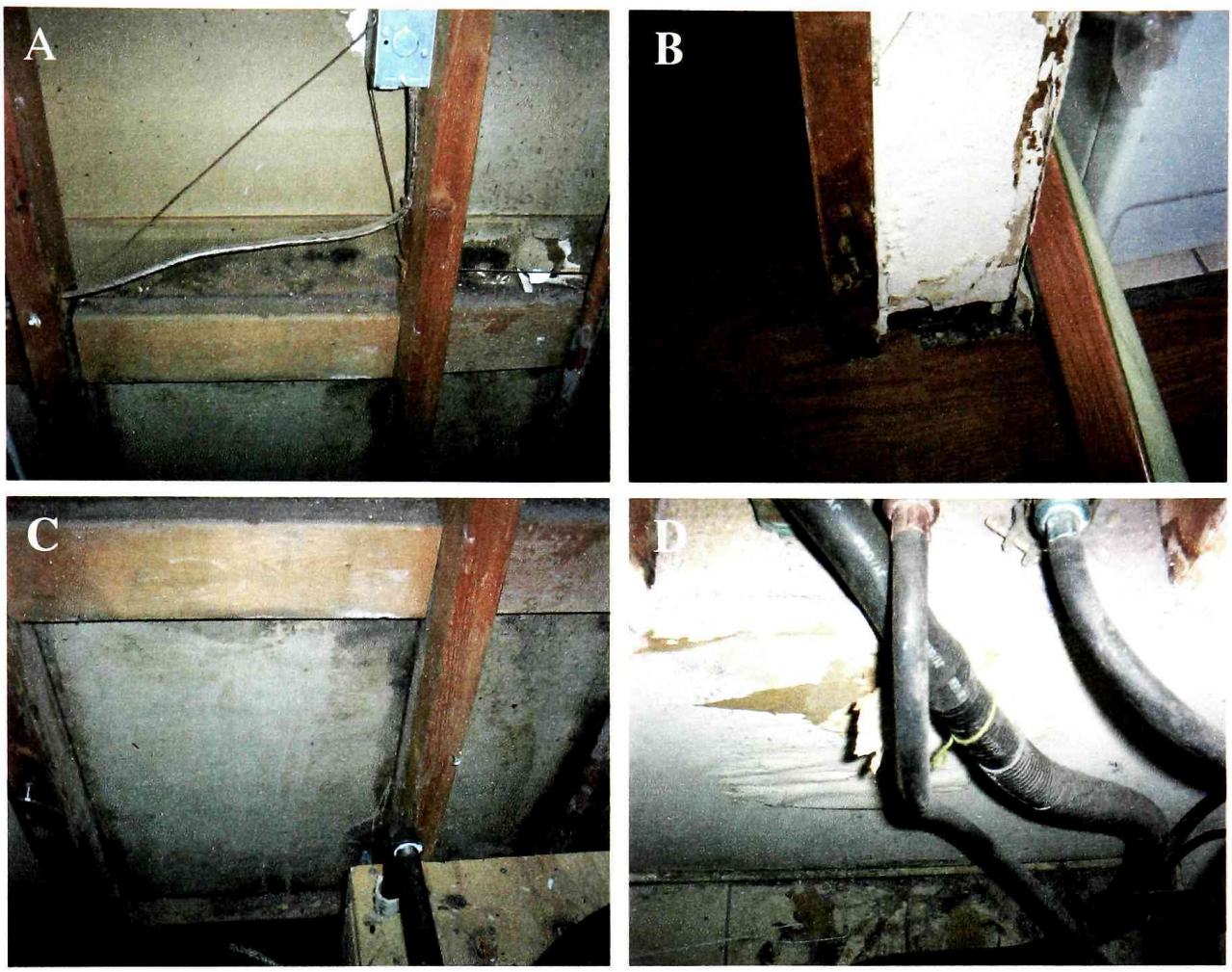


Figure C.5. Select Images from Building 6. Water damage as a result of old construction and numerous leaks was visible throughout this building, but was most evident in the kitchen (B and D) and garage (A and C). Visible *Stachybotrys* growth was not identified, but *Chaetomium* growth was heavy.

Table C.6. Airborne conidia types and counts isolated from Building 7* for each sampling time and condition.

Room[a]	Sampling Time and Conditions[b]	Airborne Fungal Genera - Conidia Counts per m^3 of Air[c]									Debris Count[d]
		Alt	*Asco*	*Bip*	*Clado*	*Curv*	*Fus*	*Memn*	*Nigro*	*Pen/Asp*	*Stachy*	
Hall 1	120 min - Static	0	32	0	97	0	0	0	0	1516	0	Light
	20 min - Disturbed	0	452	0	161	0	0	4387	0	3548	0	Medium
Hall 2	120 min - Static	32	65	0	65	0	0	0	0	2774	0	Light
	20 min - Disturbed	0	65	0	258	0	0	0	0	13806	0	Light
Room 253	120 min - Static	0	11387	0	0	0	0	12839	0	1258	0	Light
	20 min - Disturbed	0	6387	0	0	0	0	18323	0	2226	0	Heavy
Room 259	120 min - Static	97	65	0	129	0	0	0	0	2065	0	Light
	20 min - Disturbed	0	65	0	32	0	0	0	0	6935	0	Medium

*This building was referred to in Table 3.2 as Test 3

[a]Outside air samples were not taken for these trials due to unacceptable weather conditions (rain). Outside air samples from other buildings should be used for comparison.

[b]Rooms were sampled under static and/or disturbed conditions for the noted times. Air disturbance was accomplished using 20-inch box fans on a "high" setting. Disturbance was allowed for 5 minutes prior to starting the SpinCon collection. NA; not applicable.

[c]5-minute Allergenco spore traps were taken to assess airborne fungal conidia types and concentrations as well as to qualitate the amount of debris present. For static conditions, samples were taken just prior to SpinCon collection. For disturbed conditions, Allergencos were started 10 minutes prior to the end of the sampling period. Key: *Alt - Alternaria*; Asco - Ascospores (most likely *Chaetomium*, but unable to confirm); *Bip - Bipolaris*; *Clado - Cladosporium*; *Curv - Curvularia*; *Fus - Fusarium*; *Memn - Memnoniella*; *Nigro - Nigrospora*; *Pen/Asp - Penicillium/Aspergillus*-like (unable to confirm); *Stachy – Stachybotrys*

[d]Debris was defined as non-identifiable particles and were qualitated based on the approximate percentage of the viewed field covered by such particles: Very Light (<20%), Light (21-40%), Medium (41-60%), Heavy (61-80%), and Very Heavy (>80%).

Figure C.6. Select Images from Building 7. Mold and water damage were heavy throughout this building. Hidden mold was present as demonstrated by growth behind wall papering and paneling (A-D). Visible contamination and water damage were also evident (E-F). Fungal growth seen in these images was not of *Stachybotrys*, but primarily of *Memnoniella*, an organism related to *Stachybotrys*, but one that does not produce the highly toxic macrocyclic trichothecene mycotoxins.

Table C.7. Airborne conidia types and counts isolated from Building 8 for each sampling time and condition.

Room[a]	Sampling Time and Conditions[b]	Airborne Fungal Genera - Conidia Counts per m^3 of Air[c]									Debris Count[d]
		Alt	*Asco*	*Bip*	*Clado*	*Curv*	*Fus*	*Memn*	*Nigro*	*Pen/Asp*	*Stachy*	
OSA	NA	355	258	0	1258	0	0	0	0	2548	0	Light
Closet	24 hours	32	129	0	1387	0	0	0	0	1867	0	Light
	48 hours	161	3710	0	807	0	0	0	0	10774	0	Light
	72 hours	32	129	0	1871	0	0	0	0	1677	0	Light

[a]OSA (Outside air) was considered normal and served as a control for sampling.

[b]This room was sampled using a modified Andersen High Volume PUF Sampler as described in the text under static conditions for the noted times. NA; not applicable.

[c]5-minute Allergenco spore traps were taken to assess airborne fungal conidia types and concentrations as well as to qualitate the amount of debris present. Samples were taken just prior to the Andersen collection. Key: *Alt - Alternaria*; Asco - Ascospores (most likely *Chaetomium*, but unable to confirm); *Bip - Bipolaris*; *Clado - Cladosporium*; *Curv - Curvularia*; *Fus - Fusarium*; *Memn - Memnoniella*; *Nigro - Nigrospora*; *Pen/Asp - Penicillium/Aspergillus*-like (unable to confirm); *Stachy – Stachybotrys*

[d]Debris was defined as non-identifiable particles and were qualitated based on the approximate percentage of the viewed field covered by such particles: Very Light (<20%), Light (21-40%), Medium (41-60%), Heavy (61-80%), and Very Heavy (>80%).

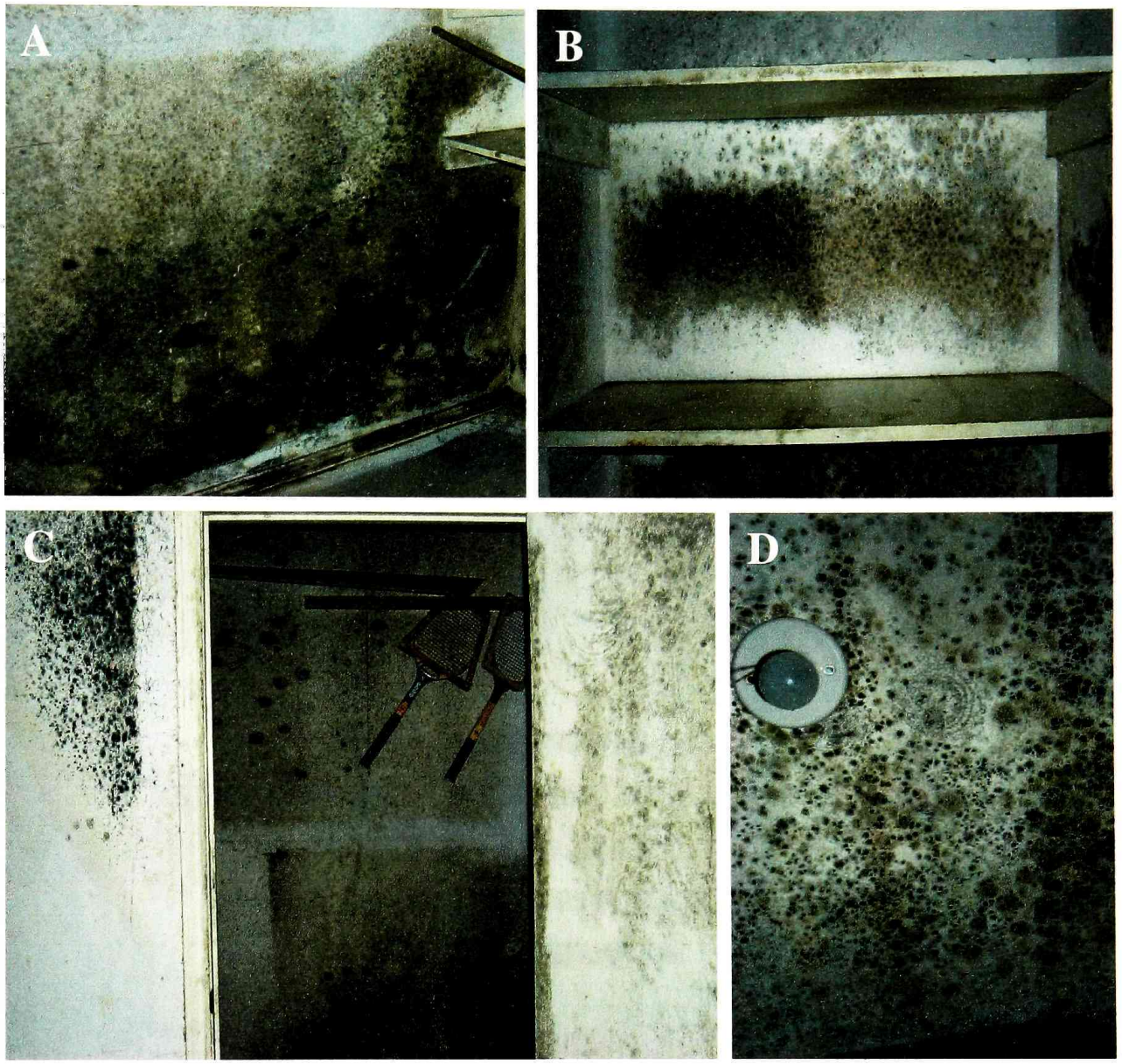


Figure C.7. Select Images from Building 8. This building suffered major water damage and ensuing fungal contamination (A-D). This room is a good demonstration of how fungi can grow together. Numerous genera were identified of which *Stachybotrys* growth was the heaviest. This storage closet was most heavily contaminated area and was chosen for sampling using the Andersen PUF High Volume Air Sampler. Virtually every surface of this room demonstrated fungal growth and thus served as an ideal sampling area.

APPENDIX D

SUPPLEMENTARY BUILDING DATA: ELISA PERCENT INHIBITIONS AND TRICHOTHECENE EQUIVALENTS FOR EACH SAMPLED AREA

Table D.1. Air sampling analyses from SpinCon-sampled *Stachybotrys chartarum*-contaminated indoor environments.

Test Building	Sample Label	Dimensions[a]	Sampling Time and Conditions[b]	Average ELISA % Inhibition[c]	Average Trichothecene Equivalent[d]	Average Trichothecene Equivalents/m^3 of Sampled Air[e]
1	Television Room	20 x 25 x 10	120 min Static	62.5 ± 1.9	2.0 ± 0.3	36.7 ± 4.7*
			120 min Disturbed	32.4 ± 0.5	0.42 ± 0.01	7.7 ± 0.2*
			30 min Disturbed	63.5 ± 2.9	2.2 ± 0.4	159.3 ± 32.1*
			10 min Disturbed	74.2 ± 1.3	5.3 ± 0.7	1172.0 ± 145.2*
	Upstairs Bedroom	20 x 25 x 8	120 min Static	21.0 ± 1.9	0.28 ± 0.02	5.2 ± 0.3
			120 min Disturbed	10.2 ± 3.5	0.20 ± 0.02	3.7 ± 0.4
			30 min Disturbed	18.1 ± 2.0	0.25 ± 0.02	18.8 ± 1.3*
			10 min Disturbed	9.0 ± 4.3	0.19 ± 0.02	42.9 ± 5.1
	Kitchen	25 x 30 x 8	120 min Static	17.2 ± 3.0	0.25 ± 0.02	4.6 ± 0.4
			30 min Disturbed	17.5 ± 0.6	0.25 ± 0.00	18.3 ± 0.4*
			10 min Disturbed	30.2 ± 3.0	0.39 ± 0.04	85.9 ± 9.4
2	Kitchen	20 x 20 x 8	120 min Static	50.8 ± 3.5	0.98 ± 0.17	18.1 ± 3.2*
			120 min Disturbed	30.8 ± 3.6	0.40 ± 0.05	7.4 ± 1.0*
			30 min Disturbed	17.3 ± 4.0	0.25 ± 0.03	18.4 ± 2.4*
	Main Entry Room	20 x 25 x 8	120 min Static	16.8 ± 1.4	0.24 ± 0.01	4.5 ± 0.2
			120 min Disturbed	21.4 ± 1.4	0.28 ± 0.01	5.2 ± 0.3
			30 min Disturbed	49.7 ± 3.8	0.93 ± 0.20	68.8 ± 14.7*
			10 min Disturbed	50.4 ± 0.6	0.95 ± 0.03	210.4 ± 7.0*
3	Laundry Room	6 x 8 x 8	120 min Static	19.0 ± 2.1	0.26 ± 0.02	4.8 ± 0.3
			10 min Agg 1	95.9 ± 0.4	Above scale	Above scale*
			10 min Agg 2	96.0 ± 0.3	Above scale	Above scale*
			10 min Agg 3	96.1 ± 0.2	Above scale	Above scale*
			10 min Agg 1 (1:10)	59.1 ± 3.1	1.6 ± 0.3	355.0 ± 65.6*
			10 min Agg 2 (1:10)	69.5 ± 1.5	3.4 ± 0.5	756.7 ± 99.5*
			10 min Agg 3 (1:10)	75.7 ± 1.0	6.2 ± 0.7	1368.1 ± 144.9*
4	Enclosed Living Room Area	12 x 4 x 8	30 min Agg	63.6 ± 0.9	2.1 ± 0.2	157.6 ± 10.8*
5	Front Bathroom	10 x 8 x 8	120 min Static	38.5 ± 2.3	0.54 ± 0.05	10.0 ± 1.0*
	Back Bathroom			39.4 ± 0.9	0.56 ± 0.02	10.3 ± 0.4*

Table D.1. Continued.

Test Building	Sample Label	Dimensions[a]	Sampling Time and Conditions[b]	Average ELISA % Inhibition[c]	Average Trichothecene Equivalent[d]	Average Trichothecene Equivalents/m^3 of Sampled Air[e]
6	Garage	15 x 25 x 8	120 min Static	38.3 ± 5.2	0.54 ± 0.12	10.1 ± 2.1*
			10 min Disturbed	26.1 ± 1.6	0.33 ± 0.02	73.6 ± 4.1
	Kitchen	15 x 20 x 8	120 min Static	31.4 ± 5.3	0.41 ± 0.08	7.6 ± 1.4*
			10 min Disturbed	24.5 ± 3.4	0.32 ± 0.04	70.1 ± 8.3
7	Hall 1	8 x 90 x 8	120 min Static	10.2 ± 2.4	0.20 ± 0.01	3.7 ± 0.3
			20 min Disturbed	7.9 ± 3.1	0.19 ± 0.02	20.7 ± 1.9
	Room 253	15 x 15 x 8	120 min Static	4.3 ± 2.2	0.17 ± 0.01	3.1 ± 0.2
			20 min Disturbed	9.6 ± 2.2	0.20 ± 0.01	21.7 ± 1.4
	Hall 2	8 x 90 x 8	120 min Static	4.5 ± 4.2	0.17 ± 0.02	3.2 ± 0.4
			20 min Disturbed	6.2 ± 0.9	0.18 ± 0.00	19.7 ± 0.5
	Room 259	15 x 15 x 8	120 min Static	15.3 ± 1.5	0.23 ± 0.01	4.3 ± 0.2
			20 min Disturbed	9.7 ± 1.6	0.20 ± 0.01	21.7 ± 1.0

[a]Length x width x height in feet.

[b]Rooms were sampled under static and/or disturbed conditions for the noted times. Air disturbance was accomplished using 20-inch box fans on a "high" setting. Disturbance was allowed for 5 minutes prior to starting the SpinCon collection. Certain areas were sampled during aggressive sampling and are noted ("Agg").

[c]Means ± standard deviations. Values are based on PBS alone (average of 8 separately run samples). Values represent triplicate wells.

[d]In ng/ml. Means ± standard deviations are shown. Values were derived from an ELISA-based macrocyclic trichothecene standard curve (Figure 2.3). Values represent triplicate wells.

[e]In picograms. Means ± standard deviations are shown. Estimated values are based on the average trichothecene equivalents for the entire collected sample, collection time, and flow rate of the SpinCon. For example, a total of 54 m^3 of air was collected for each two-hour sample. Given a final working volume of 1 milliliter, trichothecene concentrations were then estimated from values obtained from the ELISA testing. Values represent triplicate wells. Values determined as significantly different ($P<0.05$) than control environments sampled in a similar manner are noted with an *. Because 30 minute disturbed sampling was not performed in control environments, these samples were compared to environments sampled for 120 minutes under static conditions.

Table D.2. Air sampling analyses from SpinCon-sampled control environments.

Control Building	Sample Label	Dimensions	Sampling Time and Conditions[a]	Average ELISA % Inhibition[c]	Average Trichothecene Equivalent[d]	Average Trichothecene Equivalents/m^3 of Sampled Air[e]
1	Room 1	15 x 20 x 10	120 min Static	21.5 ± 2.6	0.28 ± 0.02	5.3 ± 0.5
	Room 2 (Trial 1)	30 x 20 x 10		1.7 ± 1.2	0.16 ± 0.01	2.9 ± 0.1
	Room 2 (Trial 2)			5.2 ± 3.2	0.17 ± 0.02	3.2 ± 0.3
	Room 2 (Trial 3)			1.8 ± 3.1	0.15 ± 0.02	2.8 ± 0.4
	Room 2 (Trial 4)			18.4 ± 2.0	0.26 ± 0.02	4.7 ± 0.3
	Room 2 (Trial 5)			0.14 ± 0.24	0.14 ± 0.01	2.6 ± 0.2
	Room 3	20 x 20 x 10		20.0 ± 1.8	0.27 ± 0.02	5.0 ± 0.3
	Room 4 (Trial 1)	20 x 20 x 10		6.6 ± 4.4	0.18 ± 0.02	3.3 ± 0.4
	Room 4 (Trial 2)			13.9 ± 1.6	0.22 ± 0.01	4.1 ± 0.2
	Room 4 (Trial 3)			12.9 ± 1.8	0.21 ± 0.02	4.0 ± 0.2
	Room 4 (Trial 4)			4.6 ± 2.6	0.17 ± 0.01	3.1 ± 0.2
	Room 4 (Trial 5)			10.8 ± 3.2	0.20 ± 0.02	3.8 ± 0.4
	Room 4 (Trial 6)			0.0 ± 0.0	0.12 ± 0 02	2.2 ± 0.4
	Room 5	25 x 20 x 10		12.4 ± 1.9	0.21 ± 0.01	3.9 ± 0.2
2	Bedroom	15 x 12 x 8		11.1 ± 1.8	0.20 ± 0.01	3.8 ± 0.2
	Kitchen	25 x 15 x 10	120 min Static	17.8 ± 3.3	0.25 ± 0.03	4.7 ± 0.5
	Computer Room	20 x 20 x 8		13.2 ± 4.7	0.22 ± 0.03	4.1 ± 0.5
3	Computer Room 1	15 x 12 x 8	120 min Static	17.5 ± 0.5	0.25 ± 0.00	4.6 ± 0.1
			10 min Disturbed	4.3 ± 3.8	0.16 ± 0.03	36.1 ± 6.2
	Computer Room 2	15 x 12 x 8	120 min Static	19.7 ± 12.6	0.29 ± 0.13	5.4 ± 2.4
			10 min Disturbed	23.8 ± 15.3	0.34 ± 0.15	75.8 ± 32.8
	Bedroom	20 x 20 x 8	120 min Static	13.1 ± 2.1	0.22 ± 0.01	4.0 ± 0.3
			10 min Disturbed	14.2 ± 3.4	0.23 ± 0.02	49.9 ± 5.2

Table D.2. Continued.

Control Building	Sample Label	Dimensions[a]	Sampling Time and Conditions[b]	Average ELISA % Inhibition[c]	Average Trichothecene Equivalent[d]	Average Trichothecene Equivalents/m^3 of Sampled Air[e]
4	Bedroom 1 (Trial 1)	15 x 15 x 8	120 min Static	16.3 ± 6.5	0.25 ± 0.05	4.5 ± 0.9
	Bedroom 1 (Trial 2)		120 min Static	33.0 ± 3.2	0.43 ± 0.05	8.0 ± 1.0
			10 min Disturbed	36.7 ± 9.7	0.54 ± 0.24	120.2 ± 54.4
	Bedroom 2	15 x 15 x 8	120 min Static	59.5 ± 2.0	1.6 ± 0.2	30.0 ± 3.7
			10 min Disturbed	27.4 ± 4.2	0.35 ± 0.05	78.0 ± 11.4
	Bedroom 3	20 x 25 x 8	120 min Static	47.9 ± 10.5	0.92 ± 0.39	17.1 ± 7.2
			10 min Disturbed	35.8 ± 3.2	0.48 ± 0.06	107.4 ± 13.7
Outside Air			30 minutes	0.0	0.10 ± 0.001	7.6 ± 0.2
			60 minutes		0.09 ± 0.01	3.9 ± 0.3
			90 minutes		0.12 ± 0.01	3.0 ± 0.2
			120 minutes		0.14 ± 0.03	3.4 ± 0.7
Positive Control Setup	Sterile Ceiling Tile		10 minutes	20.8 ± 5.8	0.28 ± 0.06	62.4 ± 12.8
			30 minutes	47.4 ± 7.8	0.87 ± 0.34	64.4 ± 25.1
	Stachy Ceiling Tile		10 minutes	76.4 ± 0.6	6.7 ± 0.4	1482.5 ± 91.3
			30 minutes	87.6 ± 0.2	36.4 ± 1.6	2693.0 ± 119.9

[a]Length x width x height in feet.

[b]Rooms were sampled under static and/or disturbed conditions for the noted times. Air disturbance was accomplished using 20-inch box fans on a "high" setting. Disturbance was allowed for 5 minutes prior to starting the SpinCon collection.

[c]Means ± standard deviations. Values are based on PBS alone (average of 8 separately run samples). Values represent triplicate wells.

[d]In ng/ml. Means ± standard deviations are shown. Values were derived from an ELISA-based macrocyclic trichothecene standard curve (Figure 2.3).

[e]In picograms. Means ± standard deviations are shown. Estimated values are based on the average trichothecene equivalents for the entire collected sample, collection time, and flow rate of the SpinCon. For example, a total of 54 m^3 of air was collected for each two-hour sample. Given a final working volume of 1 milliliter, trichothecene concentrations were then estimated from values obtained from the ELISA testing.

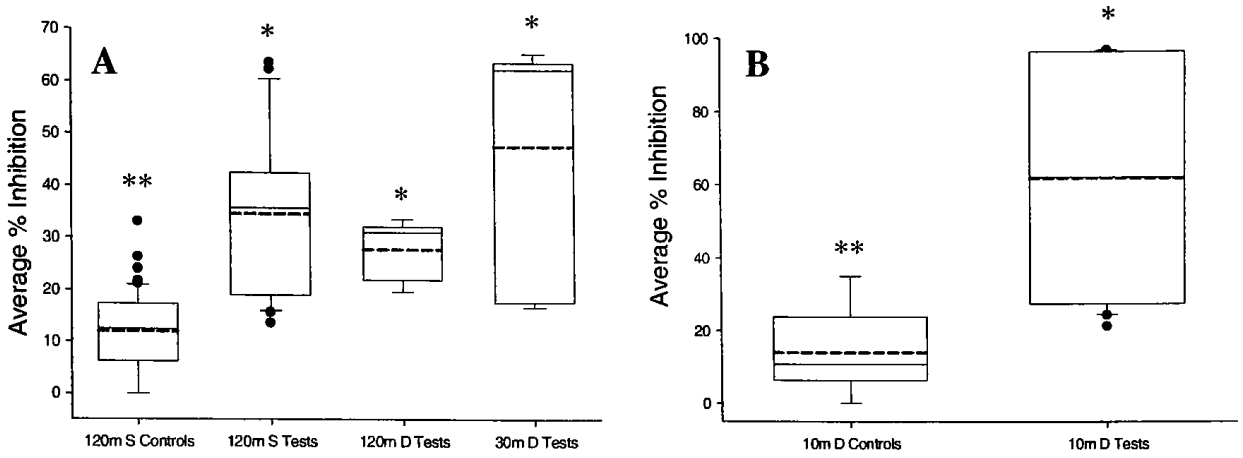


Figure D.1. Box Plot Data for Average ELISA Percent Inhibitions of Air Samples Taken in *Stachybotrys*-Contaminated and Control Indoor Environments. Percent inhibitions were calculated as described in the text. Graph A shows cumulative data obtained from 120 minute (m) control and test samples under static (S) and disturbed (D) conditions and 30-minute disturbed test environments. Medians (solid line) and means (dotted line) are shown. The 10^{th} and 90^{th} percentiles are designated by the bottom and top error bars, respectively. The 25^{th} and 75^{th} percentiles are indicated by the bottom and top of the boxes, respectively. Outliers are designated as the filled circles above and/or below the plot. Test environments were compared to control environments using a Kruskal-Wallis One Way Analysis of Variance on Ranks. Graph B shows cumulative results from control and test environments sampled for 10 minutes under disturbed conditions. Test environments were compared to controls (**) using a Mann-Whitney Rank Sum Test. Statistically significant differences ($P < 0.05$) are indicated by an *.